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(54) Title: USE OF CD26 INHIBITOR FOR THE MANUFACTURE OF A MEDICAMENT FOR THE TREATMENT OF HIV

(57) Abstract

The use of a molecule that inhibits CD26 for the preparation of a medicament for increasing the immune response of a human patient infected with HIV, by contacting the T-cells, *in vitro* or *in vivo*, with the said molecule at a concentration effective to cause T-cell proliferation, but below an amount that causes detectable cytotoxicity.

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USE OF CD26 INHIBITOR FOR THE MANUFACTURE OF A MEDICAMENT FOR THE TREATMENT OF HIV

Government Support

This work was funded in part by grant number A136696 from the National Institutes of Health. Accordingly, the United States Government may have certain rights to this invention.

Cross-Reference to Related Applications

This application claims priority from U.S. Patent Application Serial No. 08/852,394, filed on May 7, 1997, entitled TREATMENT OF HIV. The contents of this U.S Patent application are hereby expressly incorporated by reference.

Background of the Invention

This invention relates to treatment of HIV.

One of the classic markers of full-blown AIDS resulting from long-term infection with HIV-1 is a severe depletion of $CD4^+$ T-cells, which are a key component of the immune system. Attempts have been made to increase the $CD4^+$ counts of AIDS patients, and some of these efforts, notably treatment with HIV protease inhibitors, have met with considerable success. Other approaches, e.g., stimulation of the immune response by vaccination with viral peptides, have been less successful. The reasons for $CD4^+$ depletion in AIDS, and resistance of $CD4^+$ cells to stimulation by some therapies, are not fully understood.

Summary of the Invention

The invention provides a new and highly advantageous method of potentiating the immune response in HIV-infected patients, employing extremely low concentrations of compounds which, at these concentrations, act as stimulatory, rather than inhibitory, molecules. The very low concentrations employed according to the invention allow treatment with minimal side effects and toxicity. The specificity of the treatments according to the invention also helps avoid these adverse effects, which are seen for example, in treatment with immune stimulatory compounds such as Interleukin-2.

The invention involves the unexpected finding that certain compounds, which at relatively low concentrations (e.g., 10^{-4}-10^{-6}M) are cytotoxic to T-cells, nevertheless have immune stimulatory properties at extremely low concentrations (e.g., 10^{-10}-10^{-14}M). Even more

surprising, this stimulation occurs even though the HIV-infected patient's T-cells are otherwise unable to respond to T-cell proliferation-inducing stimuli. The effects of these low concentrations are paradoxical because T-cells from non-HIV-infected individuals, which respond to T-cell proliferation-inducing stimuli, do not seem to respond in the same fashion as T-cells from a HIV-infected patient when treated with the extremely low concentrations of compounds according to the invention.

The invention thus provides a method for treating the T-cells of a human subject infected with human immunodeficiency virus. The subject's T-cells are contacted with a molecule that inhibits CD26 and that stimulates immune function of the T-cells in an amount effective to stimulate immune function of the T-cells, said amount being below a concentration which causes detectable cytotoxicity of the T-cells. In one embodiment, the molecule stimulates proliferation of T-cells at the effective concentration. In other embodiments, the molecule stimulates the production of cytokines or increases cytotoxic T-lymphocyte or antibody activity.

The T-cells can be contacted *in vitro* or *in vivo*. In certain embodiments, the effective amount is below 10^{-8}M and may be between 10^{-10} and 10^{-16}M. Molarity is measured as a function of final concentration *in vitro* and as a function of blood concentration *in vivo*.

According to another aspect of the invention, the molecule can be administered in conjunction with a different therapeutic agent that increases the $CD4^+$ count of HIV-infected subjects. In this manner, treatment with effective amounts of the molecule according to the invention can enhance the therapeutic effect of other AIDS drugs. Specifically contemplated is use of the molecules according to the invention with non-CD26 protease inhibitors. It has been determined that the effects of the treatments according to the invention are particularly good in patients whose $CD4^+$ count is above about 400. In one embodiment, subjects may be treated with therapeutic agents or regimens to increase T-cell count to above 400 where the count initially is below 400. The subjects then are believed to be better candidates for treatment according to the invention. Thus, the invention contemplates the use of the molecules according to the invention to provide optimal combination AIDS therapies. Particularly contemplated for use in conjunction with the molecules of the invention are therapeutics which inhibit HIV replication by, for example, inhibiting reverse transcriptase or by inhibiting HIV protease activity. Exemplary therapeutics include the antiretroviral drugs: AZT (3′ azido-2′,3′

dideoxythymidine), ddI (2',3' dideoxyinosine), ddC(2',3' dideoxycytidine), ddT (2',3' didehydro-3'-deoxythymidine) and other nucleoside and non-nucleoside reverse transcriptase inhibitors and HIV-1 protease inhibitors: Indinavir, Ritonavir, Saquinavir. Combination therapies include the administration of one or more of these or other antiretroviral therapeutics, alone or together with therapeutics that are intended to treat the secondary infections associated with HIV and/or cytokines (e.g., GM-CSF, G-CSF, interferons, interleukins).

According to another aspect of the invention, the molecules of the invention are contacted with T-cells of a human subject infected with HIV, which T-cells are unable, prior to treatment according to the invention to respond normally to T-cell proliferation-inducing stimuli. In one particularly important embodiment, the molecules of the invention are administered in conjunction with an antigen. Ordinarily, such HIV-infected patients do not respond well to antigens because of an HIV-induced defect in the T-cell stimulation pathway. Use of the molecules according to the invention as adjuvants can render such T-cells responsive to stimulation or vaccination with antigens. The invention permits immunization of HIV-infected patients with antigens characteristic of HIV, antigens characteristic of other pathogens, antigens characteristic of cancer cells and the like. Peptide antigens in particular are contemplated.

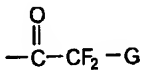
Compounds useful in the invention inhibit CD26 and stimulate proliferation of T-cells of HIV-infected subjects in the assays and at the concentrations described below. CD26 is also referred to as "dipeptidyl-aminopeptidase type-IV" or "DP-IV". CD26 is a post-prolyl cleaving enzyme with a specificity for removing Xaa-Pro (where Xaa represents any amino acid) dipeptides from the amino terminus of a polypeptide substrate.

Peptides which reportedly have demonstrated utility for inhibiting post-prolyl cleaving enzymes and which, if coupled to a reactive group, form a covalent complex with a functional group in the reactive site of a post-prolyl cleaving enzyme are described in U.S. Patent No. 4,935,493, "Protease Inhibitors", issued to Bachovchin et al. ("Bachovchin '493"); U.S. 5,462,928, "Inhibitors of Depeptidyl-aminopeptidase Type IV", issued to Bachovchin et al. ("Bachovchin '928"); U.S. 5,543,396, "Proline Phosphonate Derivatives", issued to Powers et al. ("Powers '396"); U.S. 5,296,604, "Proline Derivatives and Compositions for Their Use as Inhibitors of HIV Protease", issued to Hanko et al., ("Hanko '604"); PCT/US92/09845, "Method for Making a Prolineboronate Ester", and its U.S. priority applications (USSN 07/796,148 and 07/936,198), Applicant Boehringer Ingelheim Pharmaceuticals, Inc.

("Boehringer"). Representative structures of the transition-state analog-based inhibitors Xaa-Boro-Pro, include Lys-boroPro, Pro-boroPro, Val-boroPro and Ala-boroPro in which "boroPro" refers to the analog of proline in which the carboxylate group (COOH) is replaced with a boronyl group (B) (OH_2).

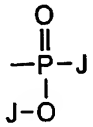
In general, the molecules useful according to the invention can have a first targeting moiety for binding a post-prolyl cleaving enzyme such as CD26 covalently coupled to a first reactive group. As used herein, a reactive group is capable of reacting with a functional group in a post-prolyl cleaving enzyme such as CD26. By reacting, it is meant that the reactive group forms a bond with a functional group of a post-prolyl cleaving enzyme such as CD26. Reactive groups that are embraced within the invention include the reactive groups referred to as group "T" in U.S. 4,935,493, "Protease Inhibitors", issued to Bachovchin, et al. These include boronate groups, phosphonate groups, and fluoroalkylketone groups. In general, it is preferred that the linkage between the carboxyl terminus of preferred peptide targeting moieties and the reactive group be in an L configuration. It is preferred that the reactive group form a covalent bond with a functional group of the active site of a reactive center of CD26; however, there is no requirement for covalent bond formation in order to form a complex between the CD26 binding molecule and CD26.

The reactive groups that are fluoroalkylketone groups have the formula:



where G is either H, F or an alkyl group containing 1 to about 20 carbon atoms and optional heteroatoms which can be N, S, or O. Additional exemplary proline phosphonate derivatives which contain a perfluoroalkyl group, a phenyl group or a substituted phenyl group and which can be used in accordance with the methods of the invention are those described in U.S. 5,543,396 (Powers '396).

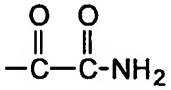
The reactive groups that are phosphonate groups have the formula:



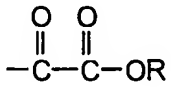
where each J, independently, is O-alkyl, N-alkyl, or alkyl (each containing about 1-20 carbon atoms) and, optionally, heteroatoms which can be n=N, S, or O. Other ketoamides, ketoacids

and ketoesters that are useful reactive groups for reacting with the reactive center of a protease are described in PCT/US91/09801, "Peptides, Ketoamides, Ketoacids, and Ketoesters", Applicant: Georgia Tech Research Corp. ("GA Tech") which claims priority to U.S. 635,287, filed December 28, 1990.

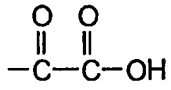
In certain embodiments, the reactive groups are selected from the groups having the formulas:



an alphaketo amide;



where R is an alkyl, or aryl group and may be substituted or unsubstituted, an alphaketo ester; and



an alphaketo acid.

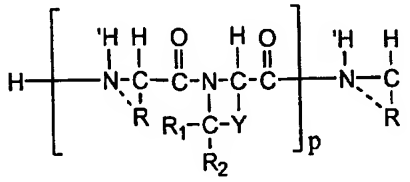
The preferred compounds having targeting moieties that are peptides which mimic the substrate binding site of DP-IV. Peptide analogs and nonpeptides or peptidomimetics also can be used as targeting moieties. Such molecules can be rationally designed based upon the known sequence of substrates of DP-IV or can be identified using combinatorial chemistry and screening assays such as are described below.

The development of phage display libraries and chemical combinatorial libraries permits the selection of synthetic compounds which mimic the substrate binding site of a protease such as CD26. Such libraries can be screened to identify non-naturally occurring putative targeting moieties by assaying protease cleavage activity in the presence and absence of the putative phage display library molecule or combinatorial library molecule and determining whether the molecule inhibits cleavage by the protease of its natural substrate or of a substrate analog (e.g., a chromophoric substrate analog which is easily detectable in a spectrophotometric assay). Those phage library and/or combinatorial library molecules which exhibit inhibition of the protease then can be covalently coupled to the reactive groups R disclosed herein and again tested to determine whether these novel molecules selectively bind

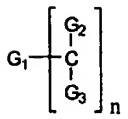
to the protease (e.g., by repeating the above-noted screening assay). In this manner, a simple, high-through-put screening assay is provided for identifying non-naturally occurring targeting moieties of the invention.

Compounds useful in the invention include, but are not limited to, compounds that inhibit CD26 and are embraced by the following formula PR, wherein P represents a targeting moiety that binds to CD26 and R represents a reactive group that reacts with a functional group in CD26, preferably a reactive center of CD26. P can be any molecule that binds CD26 including CD26 binding molecules embraced by the formula: D~A_1 -A_2 -A_3 -A_4, wherein D is independently selected from the group consisting of NH and NH_2, wherein N represents any isotope of nitrogen, wherein H represents any isotope of hydrogen; "~", independently, is selected from the group consisting of a single bond and a double bond; A_1 is selected from the group consisting of a C, a CX and an N, wherein C represents any isotope of carbon, X represents any atom that forms a single bond with carbon; each A_2, A_3, and A_4, independently, is selected from the group consisting of a CX moiety, a CXZ moiety, a CZ moiety, a NX moiety, and an O, wherein X and Z, independently are selected from the group consisting of any atom that forms a single bond and any atom that forms a double bond with C or N and wherein O represent any isotope of oxygen.

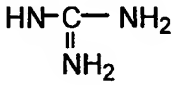
Compounds useful according to the invention also include the following of Group I or Group II. Group I has the structure:



where H represents a hydrogen: C represents a carbon; O represents an oxygen; N represents a nitrogen; each R, independently, is chosen from the group consisting of the R groups of an amino acid, including proline; each broken line, independently, represents a bond to an H or a bond to one R group, and each 'H represents that bond or a hydrogen; and p is an integer between 0 and 4 inclusive. Alternatively, Group I has the structure:

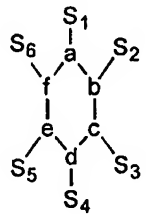


where n is between 0 and 3 inclusive, each G2 and G3 independently is H or Cl-C3 (one to three carbon atoms) alkyl, G1 is NH_3 (H_3 represents three hydrogens),

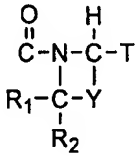


(H_2 represents two hydrogens), or NG4, where G4 is

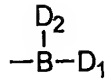
where G5 and G6 can be NH, H, or Cl-C3 alkyl or alkenyl with one or more carbons substituted with a nitrogen. G1 bears a charge, and G1 and Group II do not form a covalently bonded ring structure at pH 7.0. Group I may also have the structure:



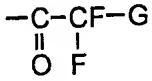
where one or two of the a, b, c, d, e, and f group is N, and the rest are C, and each S1-S6 independently is H or Cl-C3 alkyl. Group I may also include a five membered unsaturated ring having two nitrogen atoms, e.g., an imidazole ring. Group II has the structure:



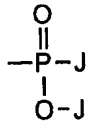
where T is a group of the formula:



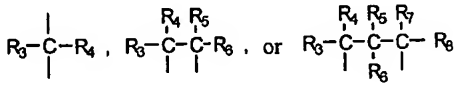
where each D_1 and D_2, independently, is a hydroxyl group or a group which is capable of being hydrolyzed to a hydroxyl group in aqueous solution at physiological pH; a group of the formula:



where G is either H, fluorine (F) or an alkyl group containing 1 to 20 carbon atoms and optional heteroatoms which can be N, S (sulfur) or O; or a phosphonate group of the formula:



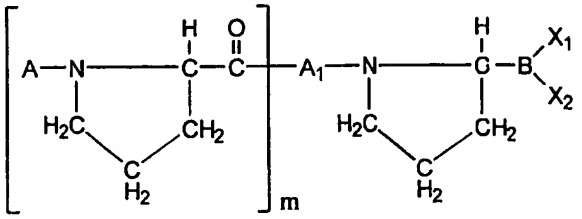
where each J, independently, is O-alkyl, N-alkyl, or alkyl. Each O-alkyl, N-alkyl or alkyl includes 1-20 carbon atoms and, optionally, heteroatoms which can be N, S, or O. T is generally able to form a complex with the catalytic side of a DP-IV. Y is:



and each R_1, R_2, R_3, R_4, R_5, R_6, R_7, and R_8, separately is a group which does not significantly interfere with site specific recognition of the inhibitory compound by DP-IV, and allows a complex to be formed with DP-IV.

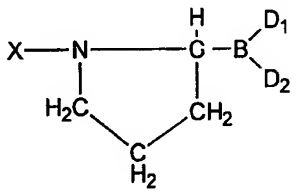
In preferred embodiments, T is a boronate group, a phosphonate group or a trifluoroalkyl ketone group; each R_1-R_8, is H; each R_1 and R_2 is H, and each Y is the CH_2-CH_2; each R is independently chosen from the R group of proline and alanine; the inhibitory compound has a binding or dissociation constant to DP-IV of at least 10^{-9}M, 10^{-8}M or even 10^{-7}M; and each D1 and D2 is, independently, F or D1 and D2 together are a ring containing 1 to 20 carbon atoms, and optionally heteroatoms which can be N, S, or O. These compounds are described in U.S. Patent No. 5,462,928, hereby incorporated by reference.

More preferably, the compound used at low concentration according to the invention has the formula:



wherein m is an integer between 0 and 10, inclusive; A and A_1 are amino acid residues such that the A in each repeating bracketed unit can be a different amino acid residue; the bonds between A and N,A_1 and C, and between A_1 and N are peptide bonds; and each X_1 and X_2 is, independently, a hydroxyl group or a group capable of being hydrolyzed to a hydroxyl group at physiological pH. These compounds are also described in U.S. Patent No. 5,462,928.

The most preferred compounds are of the formula:



where each D_1 and D_2, independently, is a hydroxyl group or a group which is capable of being hydrolyzed to a hydroxyl group in aqueous solution at physiological pH;

and wherein X is a targeting moiety that mimics the site of a substrate recognized and cleaved by CD26.

Other features and advantages of the invention will be apparent from the following detailed description thereof, and from the claims.

Brief Description of the Drawings

Fig. 1 is a pair of graphs showing the lymphocyte stimulatory effect of treatment of the invention on peripheral blood mononuclear cells (PBMC) from HIV-infected and uninfected patients. Fig. 1A shows the effect of the compound on T-cell proliferation *in vitro* for PBMC from an HIV-1^+ individual and Fig. 1B shows the effect of the compound on T-cell proliferation *in vitro*

on PBMC from an HIV-1$^-$ individual. Each of Figs. 1A and 1B illustrate a representative experiment out of a total of ten experiments.

Fig. 2 is a graph illustrating the T-cell stimulatory effects of two inhibitory compounds used according to the invention (date of experiment: 3/9/95; Patient ID no:1655185; CD4 antibody count:760; and number of cells/well: 0.4 x 10^6).

Fig. 3 is a histogram demonstrating that an inhibitor according to the invention induces, at higher doses than in the invention, dose-dependent apoptosis in both CD26$^+$ and CD26$^-$ populations of PBMC. The CD26$^+$ and CD26$^-$ PBMC populations were found to be equally susceptible to DPPIV inhibitor induced death. PBMC were stained with the anti-CD26 monoclonal antibody, 4 EL, and then sorted into CD26$^+$ and CD26$^-$ populations using a facstar plus dual lasar flow cytometry. The cells expressing the highest level (5%) of CD26 and the cells expressing the lowest level (bottom 10%) of CD26 were isolated as the CD26$^+$ and CD26$^-$ populations respectively. The purity of the populations as examined by staining with the anti-CD26 monoclonal antibody, 134-2C2, is >90%. The CD26$^+$ and CD26$^-$ populations were cultured overnight in the presence or absence of various concentrations of VBP. The amount of death induced by VBP treatment was determined by 7AAD flow cytometry analysis. Data represent mean of death from duplicate samples +/-SD.

Fig. 4 is a graph illustrating a stimulatory effect of an inhibitor according to the invention on PBMC *in vitro*, showing the correlation with CD4$^+$ counts. The data are plotted as the natural log of the stimulation index (vertical dimension) versus the natural log of the CD4$^+$ count of the patient (horizontal dimension)(71 patients total; P=<0.0001; RR=2.04 (1.5-2.9)).

Figs. 5 and 6 are graphs showing T-cell stimulatory effect on lymphocytes from HIV-infected patients by a CD26 inhibitor according to the invention, and giving results for control compounds.

Detailed Description of the Invention

In general, the compounds useful according to the invention are capable of stimulating immune function of T-cells from HIV-infected human subjects. Stimulation of immune function is preferably determined by measuring whether T-cells proliferate in response to treatment according to the invention. Assays for proliferation are described below. Stimulation of immune function also can be determined by detecting an increase in cytokine release or by detecting an increase in cytotoxic T-lymphocyte or antibody activity, using routine, commercially available

assays and/or reagents.

The molecules are contacted with T-cells in amounts effective to stimulate immune function. Effective amounts are those which cause detectable changes in any one or more of the foregoing parameters. Thus, for example, an effective amount can be that amount which induces measurable proliferation of an HIV-infected subject's T-cells. The effective amounts according to the invention, however, are amounts below a concentration which causes detectable cytotoxicity of a patient's T-cells. As used herein, detectable cytotoxicity means no more than 20%, preferably no more than 10% and most preferably no more than 5% cytotoxicity as compared to controls. At such levels, the proliferation effects of the compounds will outweigh the cytotoxic effects.

An important aspect of the invention is the discovery that relatively high concentrations (e.g., 10^{-4}-10^{-6} M) of certain compounds useful according to the invention cause substantial cytotoxicity of T-cells. For example, using Val-boroPro, greater than 80% toxicity was observed *in vitro* at a concentration of 10^{-4}M and approximately 50% cytotoxicity was observed at a concentration of 10^{-6}M. Although cytotoxicity decreased with decreasing concentrations of Val-boroPro, it was not expected that there would be such substantial cytotoxicity at concentrations of 10^{-6}. Unexpectedly, however, stimulatory properties increased substantially at concentrations significantly below those concentrations which were cytotoxic. Maximum stimulation was observed at concentrations of 10^{-12}-10^{-14}M. Thus, the compounds according to the invention can be applied at extremely low concentrations *in vitro*, or *in vivo*, thereby avoiding any detectable cytotoxicity yet achieving the highest levels of immune stimulation.

The foregoing observation is important when considering treatments of T-cells of HIV-infected subjects, particularly *in vivo*. AIDS subjects already suffer drastic reductions in T-cells. It could be catastrophic to administer compounds which would destroy the relatively meager amount of T-cells present in an AIDS patient. The present invention is based upon the discovery that concentrations of certain compounds can be applied far below those concentrations that would be cytotoxic while achieving the highest levels of immune stimulation capable of being induced by such compounds.

In selecting effective amounts for *in vivo* treatment, concentrations can be selected far outside the range that would be expected to cause cytotoxicity. This concentration then could be increased, all the while measuring T-cell count, until maximum stimulation is observed (as in a bell curve). Maximum stimulation will occur, as shown in the Examples below, long before cytotoxic levels are achieved. Thus, effective *in vivo* dosing can be accomplished without risk to the patient.

In selecting amounts for *in vitro* treatment, assays such as those shown in the Examples below can be used. One such assay employs the candidate compound at very low concentrations, in a test designed to determine whether at such concentrations the compound can stimulate proliferation of PBMC from HIV-infected patients *in vitro*. As is shown in the Examples, stimulation can be measured by, e.g., incorporation of a labeled nucleotide. *In vitro* screening also can be used to assist in developing *in vivo* effective amounts.

Where *in vitro* treatment is contemplated, cells are obtained from a subject, stimulated *in vitro*, and then returned to the subject in a conventional manner. Where *in vivo* treatment is contemplated, the compounds useful according to the invention are administered in a conventional manner, as described in greater detail below.

The molecules useful according to the invention can be administered *in vivo* as concurrent therapy or in combination with other AIDS therapeutics. While not wishing to be bound by any theory of the invention, it is believed that the compounds of the invention will beneficially stimulate proliferation of *non-infected* T-cells in the HIV-infected patient. The concurrent treatments then can be used to bring about proliferation of non-infected T-cells, which in turn can be stimulated beneficially according to the methods of the present invention. The fact that, in T-cells treated according to the invention *in vitro* no increase in the level of the HIV protein p24 is observed, may indicate that the T-cells which are infected with HIV are not stimulated by the low dose treatment of the invention. The inventors explicitly, however, do not intend to be bound by this theory of the invention.

Particularly preferred compounds useful according to the invention are described in the Examples below. The invention, however, embraces the specific compounds described above. In order to identify additional compounds useful according to the invention, simple screening assays can be employed. First, compounds rationally designed based upon the natural substrate of a post-prolyl cleaving enzyme or libraries of compounds prepared by combinatorial chemistry or phage display are screened for their ability to inhibit CD26. CD26 and methods of purifying CD26 are well-known to those of ordinary skill in the art. Torimoto, Y., et al., *Molecular Immunol.* 29:183-192 (1992). Assays which measure, for example, the ability of a molecule to interfere with CD26 cleavage of a standard fluorescent-labeled substrate can be employed. Once a candidate compound that inhibits CD26 is identified, then the compound is tested over a wide range of concentrations (e.g., 10^{-4}-10^{-16} M) to determine the ability of the compound to stimulate immune function of T-cells from HIV-infected patients. Methods for performing this test are described in the Examples

below, which involve measurement of proliferation.

In the screening assays described in the previous paragraph, a reporter substrate such as Ala-Pro-AFC, which contains a proline in the penultimate position, can be employed. Alternatively, a colorimetric assay can be carried out using as a substrate Gly-Pro-PNA. The choice of terminal amino acid is not critical, provided that the substrate contain a free terminal amino group. A fluorescence spectrometer then can be used to measure cleavage of the reporter substrate. Multiple samples can be run, with or without a test compound. The samples are placed in a cuvette, inserted into a fluorescent spectrometer, and enzymatic activity is measured as the accumulation of fluorescence intensity (i.e., substrate cleavage product) over time (e.g., 1 minute). A compound is identified as a good candidate if the accumulative fluorescence is decreased as a result of the presence of the compound.

When methods are carried out *in vivo*, the effective amount will vary with the particular condition being treated, the age and physical condition of the subject being treated, the severity of the condition, the duration of the treatment, the nature of the concurrent therapy (if any), the specific route of administration and the like factors within the knowledge and expertise of the health practitioner. For example, an effective amount can depend upon the degree to which an individual has abnormally depressed levels of T-cells.

Generally, doses of active compounds would be from about 0.001 mg/kg per day to 1000 mg/kg per day. It is expected that doses ranging from .01-10 mg/kg will be suitable, preferably orally and in one or several administrations per day. Lower doses will result from other forms of administration, such as intravenous administration. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits, provided that toxic levels are not administered. Multiple doses per day are contemplated to achieve appropriate systemic levels of compounds.

When administered, the pharmaceutical preparations of the invention are applied in pharmaceutically-acceptable amounts and in pharmaceutically-acceptable compositions. Such preparations may routinely contain salt, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When used in medicine, the salts should be pharmaceutically-acceptable, but non-pharmaceutically-acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically- and pharmaceutically-acceptable salts include, but are not limited to,

those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts.

The molecules may be combined, optionally, with a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

The pharmaceutical compositions may contain suitable buffering agents, including: acetic acid in a salt; citric acid in a salt; boric acid in a salt; and phosphoric acid in a salt.

The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the anti-inflammatory agent, which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed using synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulation suitable for oral, subcutaneous, intravenous, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular drug selected, the severity of the condition being treated and the dosage required for therapeutic efficacy. The methods of the invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that

produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, topical, nasal, interdermal, or parenteral routes. The term "parenteral" includes subcutaneous, intravenous, intramuscular, or infusion. Intravenous or intramuscular routes are not particularly suitable for long-term therapy and prophylaxis. They could, however, be preferred in emergency situations. Oral administration will be preferred for prophylactic treatment because of the convenience to the patient as well as the dosing schedule.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active agent into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active agent. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the active agent, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include polymer base systems such as poly(lactide-glycolide), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polyanhydrides. Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Patent 5,075,109. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono-di- and tri-glycerides; hydrogel release systems; sylastic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like. Specific examples include, but are not limited to: (a) erosional systems in which the active agent is contained in a form within a matrix such as those described in U.S. Patent Nos. 4,452,775, 4,667,014, 4,748,034 and 5,239,660 and (b) diffusional systems in which the active agent permeates at a controlled rate from a polymer such as described in U.S. Patent Nos. 3,832,253 and 3,854,480. In addition, pump-based hardware

delivery systems can be used, some of which are adapted for implantation.

Use of a long-term sustained release implant may be particularly suitable for treatment of chronic conditions. Long-term release, as used herein, means that the implant is constructed and arranged to deliver therapeutic levels of the active ingredient for at least 30 days, and preferably 60 days. Long-term sustained release implants are well-known to those of ordinary skill in the art and include some of the release systems described above.

Certain families of molecules useful according to the invention are described in U.S. Patent Nos. 4,935,493, 5,462,928, and in U.S. Application Serial No. 07/923,337, corresponding to PCT Application No. WO94/03055. One of the families of molecules described in these patents may be referred to as the Xaa-Boro-Pro molecules, exemplified by Ala-boroPro, Pro-boroPro, and Gly-boroPro. Two of these compounds, used in the Examples described below, are Lys-Boro-Pro ("KBP") and Val-Boro-Pro ("VBP").

EXAMPLES

Peripheral blood mononuclear cells (PBMC) were obtained by standard methods from HIV-infected individuals, and from uninfected individuals. Varying dosages of KBP or VBP were contacted with the PBMC *in vitro*, and stimulation of proliferation was measured by incorporation of ^{3}H thymidine (cpm). The results of these experiments are shown in Fig. 1: very low doses of the Val-Boro-Pro and Lys-Boro-Pro stimulated proliferation of PBMC from HIV-infected patients, but not PBMC from uninfected patients.

As shown in Fig. 1, at no concentration tested of the compounds did they affect the PBMC from uninfected individuals. The compounds, at moderate concentrations, also did not cause proliferation of PBMC from HIV-infected individuals, but did cause marked proliferation at very low concentrations (10^{-9} and 10^{-10}M). The same results are shown in Figs. 5 and 6 which also present data for two control compounds OKT3 and PHA, both of which are non-specific mitogens.

Fig. 2 is a histogram showing that relatively low doses of Lys-boroPro and Val-boroPro (10^{-7}M) caused proliferation of PBMC of HIV-infected patients, while higher doses (10^{-4}M) did not have this effect. This is to be contrasted with Figure 3 which shows that Val-boroPro is highly cytotoxic to T-cells at concentrations of 10^{-4} and 10^{-6}M. Thus, even though there was stimulation at concentrations of 10^{-6} as shown in Fig. 2, it would be undesirable to use Val-boroPro in this range because it would cause T-cell death in patients already severely compromised with T-cell loss. As shown in Figs. 5 and 6, Val-boroPro can be used successfully outside of this cytotoxic

range, avoiding the cytotoxic effects.

Referring to Fig. 4, data are presented in a form showing that low concentrations of the compounds of the invention have less effect on the PBMC of HIV-infected patients whose $CD4^+$ counts are lower than about 400 (clinical indication for AIDS) than on the PBMC of HIV-infected patients whose $CD4^+$ counts are higher than about 400. In the graph of Fig. 5, the natural log of the stimulation index (the vertical axis) is plotted against the natural log of the $CD4^+$ count of the patients; as is shown.

The disclosures of any patents and patent applications referenced to herein are expressly incorporated by reference.

We claim:

CLAIMS

1. A method for treating the T-cells of a human subject infected with Human Immunodeficiency Virus, comprising:

contacting said T-cells with a molecule that inhibits CD26 and that stimulates immune function of said T-cells in an amount effective to stimulate immune function of the T-cells, said amount being below a concentration which causes detectable cytotoxicity of said T-cells.

2. The method of claim 1, wherein said molecule stimulates proliferation of said T-cells at said effective concentration.

3. The method of claim 1, wherein the T-cells are contacted *in vitro* and then are administered to the subject.

4. The method of claim 1, wherein the T-cells are contacted *in vivo*.

5. The method of claim 3, wherein the effective amount is below 10^{-8}M.

6. The method of claim 4, wherein the effective amount is a blood concentration below 10^{-8}M.

7. The method of claim 5, wherein the effective amount is between 10^{-10} and 10^{-16}M.

8. The method of claim 6, wherein the effective amount is a blood concentration between 10^{-10} and 10^{-16}M.

9. The method of claim 4, wherein the molecule is administered in conjunction with a different therapeutic agent that increases the $CD4^+$ count of HIV-infected patients.

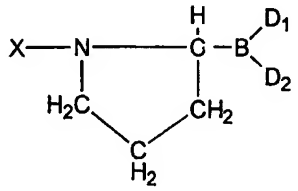
10. The method of claim 1, wherein the human subject's T-cells are unable, prior to treatment with said molecule, to respond normally to T-cell proliferation-inducing stimuli.

11. The method of claim 4, wherein the molecule is administered in conjunction with

an antigen.

12. The method of claims 1-11, wherein the molecule mimics the site of a substrate recognized by a post-prolyl cleaving enzyme and includes a reactive group that binds covalently with a functional group in a reactive center of the post-prolyl cleaving portion of CD26.

13. The method of claim 12, wherein the molecule has the formula:



wherein X is a targeting moiety that mimics the site of a substrate recognized and cleaved by CD26.

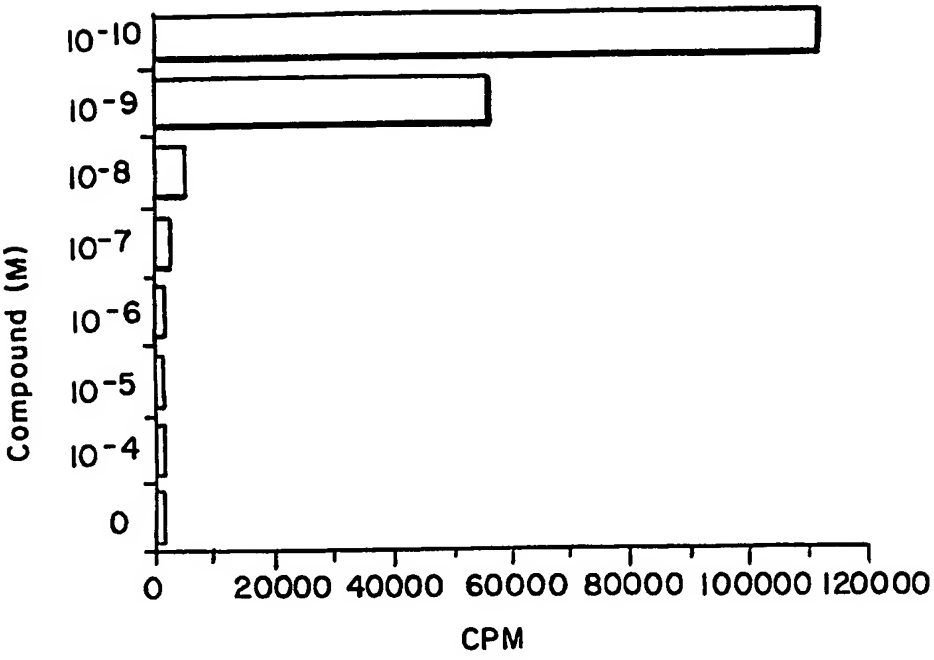

10-10
10-9
10-8
10-7
10-6
10-5
10-4
0
Compound (M)
0
20000
40000
60000
80000
100000
120000
CPM

FIG. 1A

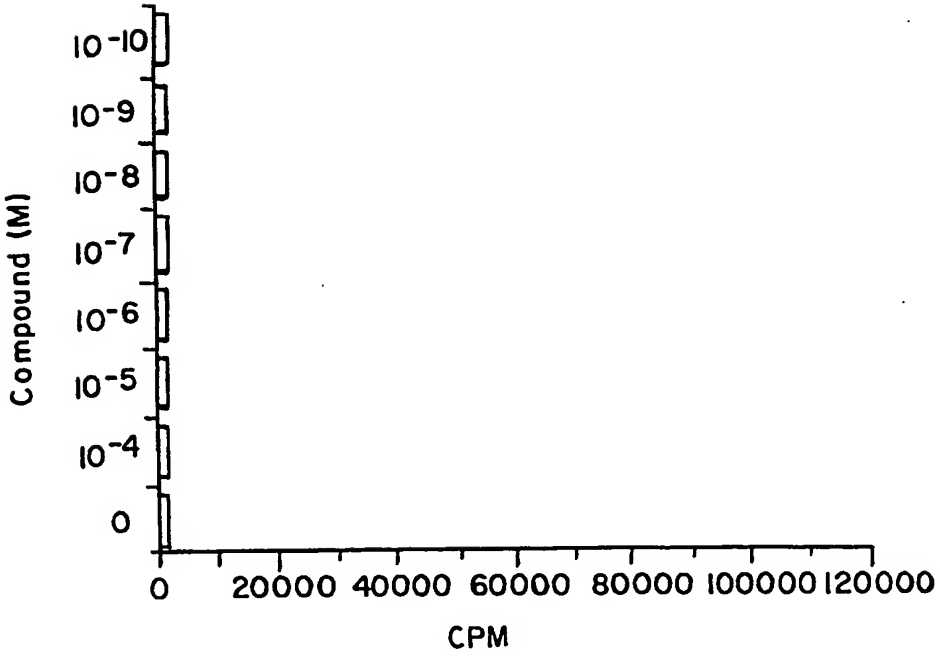

10-10
10-9
10-8
10-7
10-6
10-5
10-4
0
Compound (M)
0
20000
40000
60000
80000
100000
120000
CPM

FIG. 1B

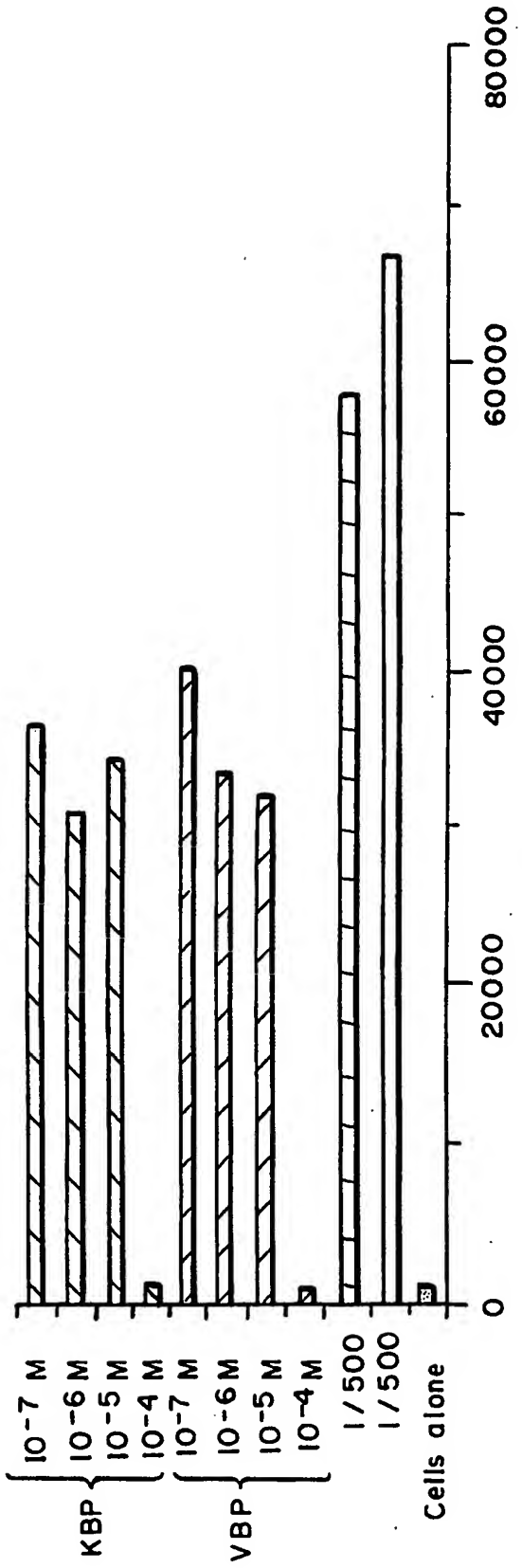

80000
60000
40000
20000
0
CPM
KBP
VBP
10⁻⁷ M
10⁻⁶ M
10⁻⁵ M
10⁻⁴ M
10⁻⁷ M
10⁻⁶ M
10⁻⁵ M
10⁻⁴ M
1 / 500
1 / 500
Cells alone

FIG. 2

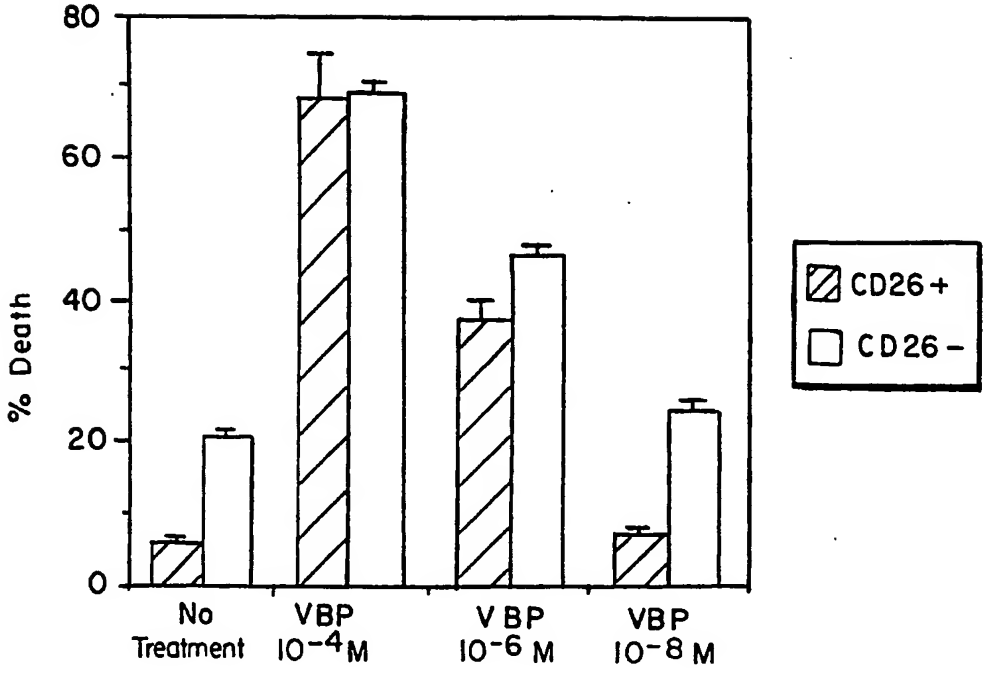

80
60
40
20
0
% Death
No Treatment
VBP 10-4 M
VBP 10-6 M
VBP 10-8 M
CD26+
CD26-

FIG. 3

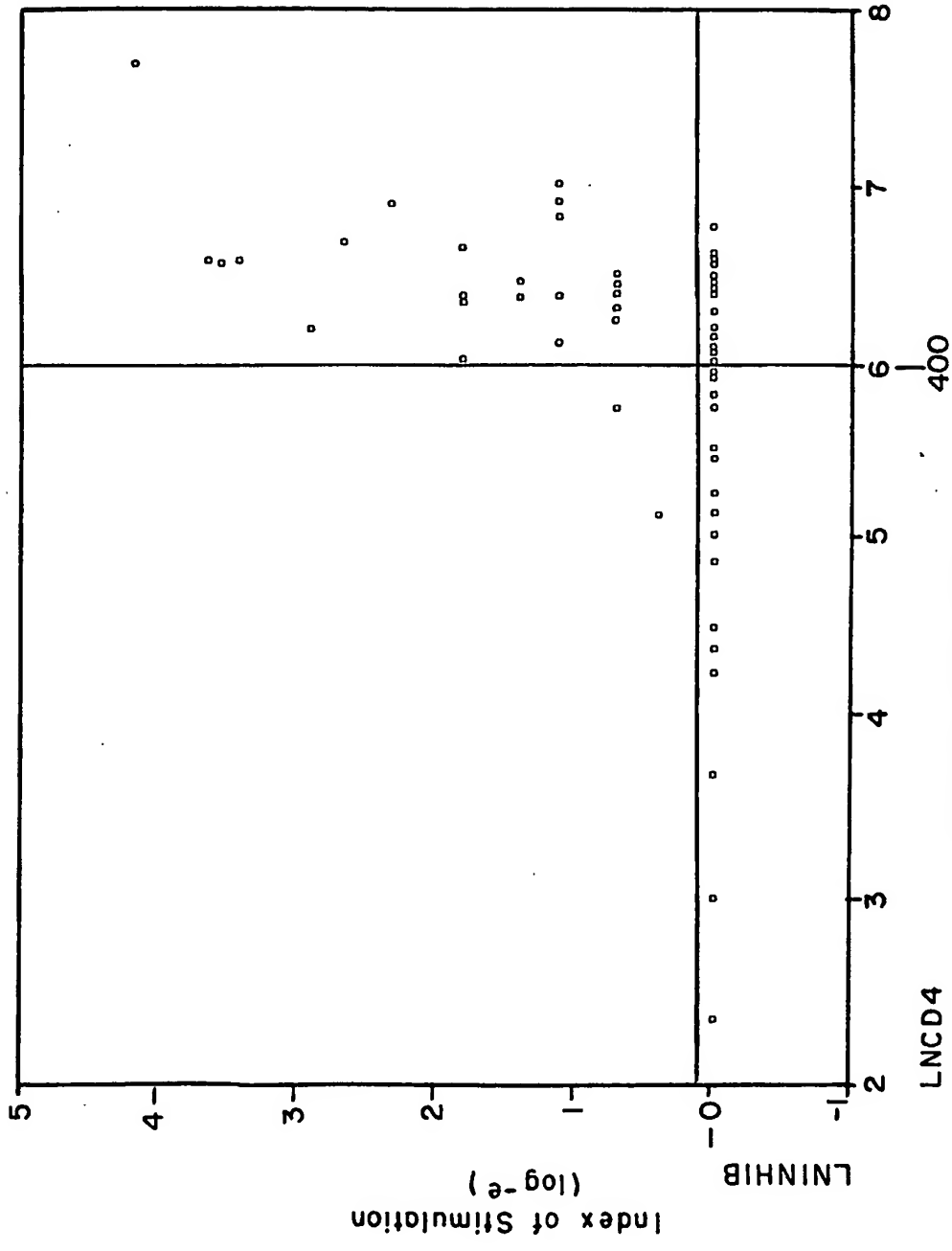

LNCD4
LINHIB
Absolute CD4 Count
(log-e)
Index of Stimulation
(log-e)
400
2
3
4
5
6
7
8
-1
0
1
2
3
4
5

FIG. 4

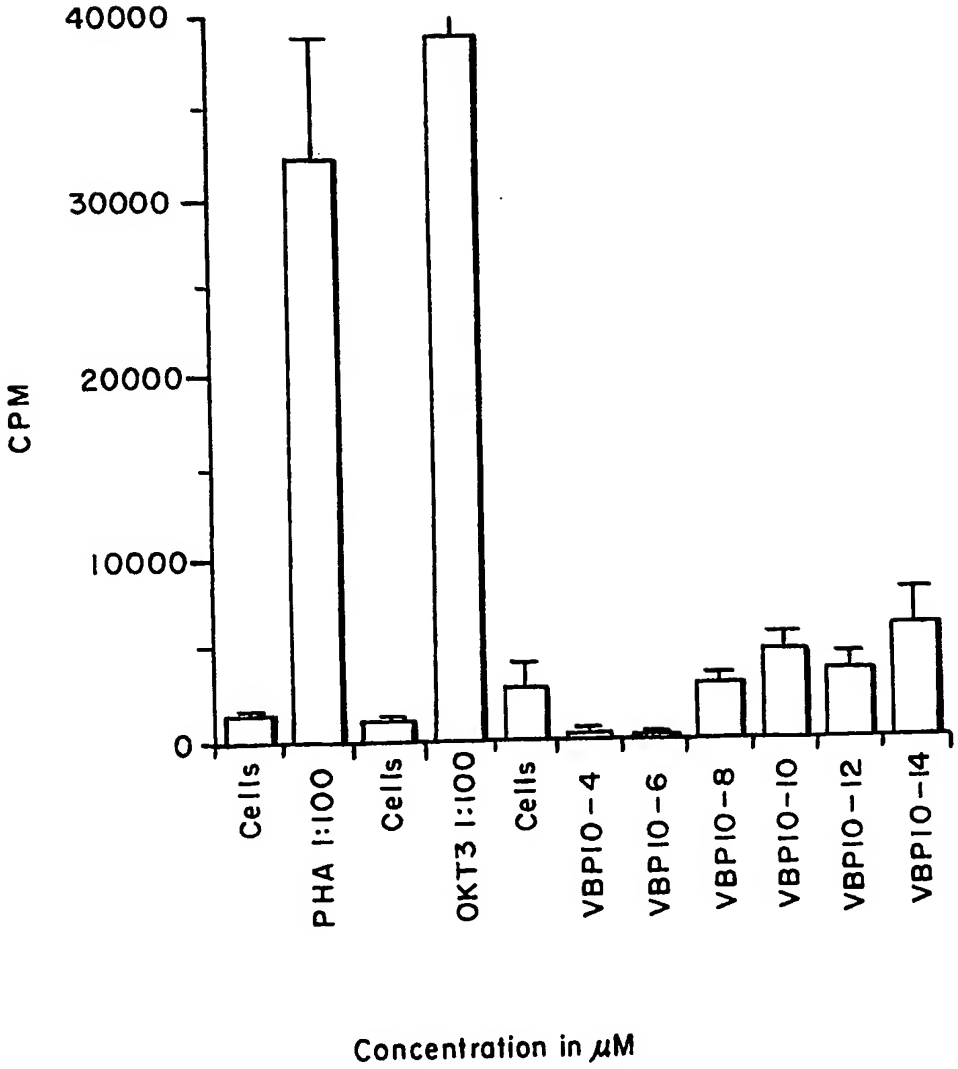

40000
30000
20000
10000
0
CPM
Cells
PHA 1:100
Cells
OKT3 1:100
Cells
VBP10-4
VBP10-6
VBP10-8
VBP10-10
VBP10-12
VBP10-14
Concentration in μM

FIG. 5

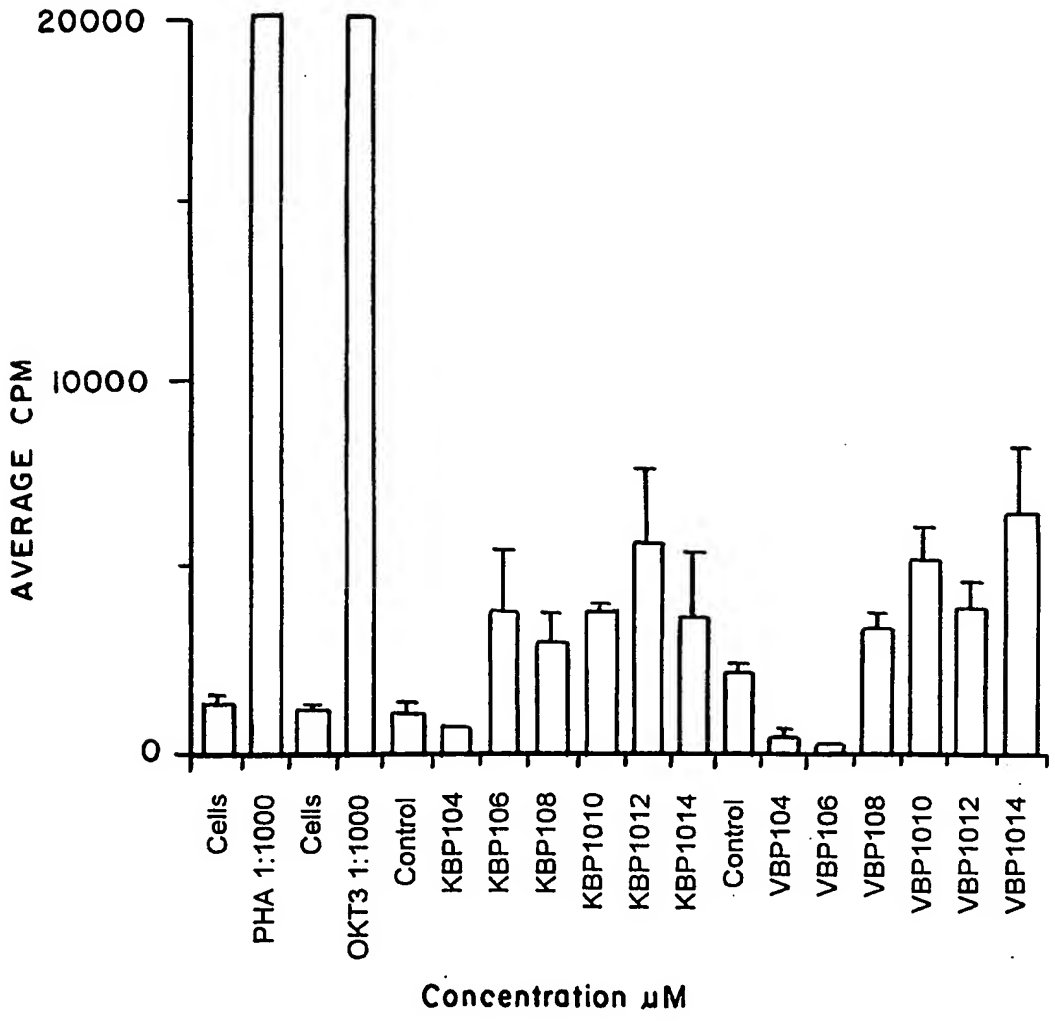

20000
10000
0
AVERAGE CPM
Cells
PHA 1:1000
Cells
OKT3 1:1000
Control
KBP104
KBP106
KBP108
KBP1010
KBP1012
KBP1014
Control
VBP104
VBP106
VBP108
VBP1010
VBP1012
VBP1014
Concentration µM

FIG. 6

INTERNATIONAL SEARCH REPORT

Inte :onal Application No
PCT/US 98/08723

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/69 A61K38/55

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 03055 A (THE GOVERNMENT OF THE UNITED STATES OF AMERICA)) 17 February 1994 cited in the application see page 1-12 see claims 1-26	1-13
X	WO 95 29691 A (GEORGIA TECH RESEARCH CO.) 9 November 1995 cited in the application see the whole document	1-13
X	WO 89 03223 A (BACHOVCHIN W.W.) 20 April 1989 cited in the application see the whole document	1-13

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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10 August 1998	01/09/1998

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INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 98/08723

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 08259 A (NEW ENGLAND MEDICAL CENTER HOSPITALS INC.) 29 April 1993 cited in the application see the whole document	1-13
X	US 5 296 604 A (MILES INC.) 22 March 1994 cited in the application see the whole document	1-13
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1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
PCT/US 98/08723

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		JP 6504547 T	26-05-1994
		US 5257901 A	02-11-1993

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Published
With international search report.

(54) Title: POTENTIATION OF THE IMMUNE RESPONSE THROUGH DELIVERY OF COMPOUNDS BINDING A CYTOPLASMIC DIPEPTIDASE

(57) **Abstract**

A method for stimulating proliferation of T–cells containing cytoplasmic post–prolyl dipeptidase activity; the method, in one aspect, involves contacting the T–cells with an organic compound at a concentration below 10^{-8}M, wherein the compound is characterized in that: (a) it is capable of crossing the membrane of T–cells to enter the cytoplasm, (b) it binds to the dipeptidase activity at a concentration of below 10^{-8}M, and thus (c) stimulates proliferation of the T–cells at that concentration.

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BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
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CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
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DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

POTENTIATION OF THE IMMUNE RESPONSE THROUGH DELIVERY OF COMPOUNDS BINDING A CYTOPLASMIC DIPEPTIDASE

Government Support

This work was funded in part by grant number A136696 from the National Institutes of Health. Accordingly, the United States Government may have certain rights to this invention.

Cross-Reference to Related Applications

This application claims priority from U.S. Patent Application Serial No. 08/852,395, filed on May 7, 1997, entitled POTENTIATION OF THE IMMUNE RESPONSE. The contents of this U.S Patent application are hereby expressly incorporated by reference.

Background of the Invention

This invention relates to treatment of viral infections using organic compounds which interact with T-cell enzymes.

One of the classic markers of full-blown AIDS resulting from long-term infection with HIV-1 is a severe depletion of $CD4^+$ T-cells, which are a key component of the immune system. Attempts have been made to increase the $CD4^+$ counts of AIDS patients, and some of these efforts, notably treatment with protease inhibitors, have met with considerable success. Other approaches, e.g., stimulation of the immune response by vaccination with viral peptides, have been less successful. The reasons for $CD4^+$ depletion in AIDS, and resistance of $CD4^+$ cells to stimulation by some therapies, are not fully understood.

Summary of the Invention

We have discovered that the activation state of human T-cells can be affected by compounds which interact with a cytoplasmic post-prolyl dipeptidase activity which has similarities to, but is distinct from , the membrane-bound T-cell serine protease CD26. The compounds useful in the invention are inhibitors of this activity, which is, in naturally-occurring T-cells in healthy individuals, involved in protection of T-cells from apoptosis, or programmed cell death. Thus, in high concentrations, the inhibitors hasten the death of T-cells, by inhibiting the protective enzyme. We have discovered, surprisingly, that at low concentrations the inhibitors exhibit a paradoxical effect: they are potent stimulators of T-cell activity in HIV-infected individuals. The concentrations of inhibitor which induce this T-cell

stimulatory response are very low (on the order of 10^{-8}-10^{-12}M), and therefore the inhibitors can be used with minimal side effects, even if, in larger doses, the inhibitors would be toxic.

Our hypothesis is that the resistance to full activation observed in T-cells of HIV-infected individuals involves a blocking of the cytoplasmic enzymatic activity discussed above. We believe that this blocking of activation, involving this cytoplasmic activity, prevents differentiation of T-cells of HIV-infected individuals into effector cells, eventually leading to T-cell death.

Thus, the invention features a method for stimulating proliferation of T-cells of a human patient suffering from a disease state characterized by the inability of the patients' T-cells to respond normally to T-cell proliferation-inducing stimuli; the method involves contacting the T-cells with an organic compound at a concentration below 10^{-8}M, wherein the compound is characterized in that it binds to the post-prolyl cleaving dipeptidase activity present in the cytoplasm of human T-cells, e.g., $CD4^+$ cells or Jurkat cells.

Treatment according to the invention can be *in vitro* or *in vivo*. In *in vivo* therapy, the enzyme-interacting compound of the invention is administered such that the blood concentration in the patient (e.g., an HIV-infected patient) is below 10^{-11}. The compounds can also be used *in vitro* at low concentrations to stimulate proliferation of non-infected, beneficial T-cells, such as $CD4^+$ cells and CTL's. In this embodiment, PBMC are isolated from a patient and incubated with a concentration of lower than 10^{-8}M of the compound, to bring about proliferation of T-cells, which are then reinfused into the patient.

We believe that administration of low concentrations of the inhibitors of the invention may have an allosteric effect such that the T-cell cytoplasmic enzyme, which is a multimeric (i.e., multiple subunit) enzyme, exhibits an increased affinity of the enzyme for its natural substrate or ligands, allowing the previously blocked T-cell to proceed to full activation, and hence survival, proliferation, and interleukin-2 production. Stimulation of the T-cell immune response in HIV-infected patients according to the invention yields increased numbers of immune effector cells, which can fight both HIV itself, and other opportunistic pathogens.

Treatment according to the invention has the advantages of specificity and low toxicity, not just because of the low concentrations of inhibitor which can be used, but also because, in T-cells of patients not infected with a virus such as HIV, the inhibitors have no discernable effect. Furthermore, treatment according to the invention advantageously does not necessarily require *in vitro* manipulation of the T-cells from HIV-infected patients. Furthermore, no

immunization is required, and treatment will be effective even where HIV proteins have mutated because the therapy targets a cellular enzyme. The fact that, in T-cells treated according to the invention *in vitro*, no increase in the level of the HIV protein p24 is observed, probably indicates that the T-cells which are infected with HIV are not stimulated by the low dose inhibitor treatment of the invention.

The invention also permits immunization of HIV-infected patients with, e.g., HIV peptides. Under normal circumstances, such patients cannot be vaccinated because of the defect in the T-cell stimulation pathway. Use of inhibitors in low doses as adjuvants can render T-cells responsive to vaccination with HIV antigens, in particular peptides.

Treatment of HIV-infected patients with low doses of inhibitors according to the invention can also enhance the activity of other AIDS drugs, in particular protease inhibitors. We have found that treatment according to the invention generally fails to bring about an increase in $CD4^+$ count in patients whose $CD4^+$ count is already very low, i.e., below about 400. In such patients, the $CD4^+$ count can be increased to above this level using known protease inhibitors, and the newly generated $CD4^+$ T-cells resulting from such treatment are particularly susceptible to the stimulatory effects of treatment according to the invention, leading to an optimal combination of AIDS therapy. Preferably, the drugs are administered orally.

The low dose administration of inhibitors of the invention can also be used to produce an adjuvant effect in HIV-negative individuals, who are to be immunized with peptides or other viral antigens; this mode of vaccination can be used for prophylaxis for HIV, as well as any other viral pathogen. Ordinarily, meaningful cytolytic T-lymphocyte ("CTL") responses, both *in vitro* and *in vivo*, have been difficult to achieve with peptide immunization. The invention should make it possible to produce significant CTL responses to viral peptides, e.g., peptides from influenza, HIV, human papilloma virus, and herpes peptides. This adjuvant effect can also be used to stimulate CTL responses to peptide antigens from other pathogens as well, e.g., pathogenic bacteria such as toxigenic *E. coli*, and protozoan pathogens such as the pathogens which are the causative agents of malaria and amoebic dysentery. The compounds, when used as adjuvants, are preferably administered orally.

The invention provides a new and highly advantageous method of potentiating the immune response in both HIV infected and uninfected patients, in methods employing extremely low concentrations of inhibitors which, at these concentrations, exhibit a paradoxical

effect (i.e., they act as stimulatory rather than inhibitory molecules, as they would at higher concentrations). The very low concentrations employed according to the invention allows treatment with minimal side reactions and toxicity. The specificity of the treatment of the invention also avoids such adverse effects, which are seen, for example, in treatment with immune stimulatory compounds such as interleukin-2.

Other features and advantages of the invention will be apparent from the following detailed description thereof, and from the claims.

Brief Description of the Drawings

Fig. 1 is a pair of graphs showing the lymphocyte stimulatory effect of treatment of the invention on peripheral blood mononuclear cells (PBMC) from HIV-infected and uninfected patients. Fig. 1A shows the effect of the compound on T-cell proliferation *in vitro* for PBMC from an HIV-1$^+$ individual and Fig. 1B shows the effect of the compound on T-cell proliferation *in vitro* on PBMC from an HIV-1$^-$ individual. Each of Figs. 1A and 1B illustrate a representative experiment out of a total of ten experiments.

Fig. 2 is a graph illustrating the T-cell stimulatory effects of two inhibitory compounds used according to the invention (date of experiment: 3/9/95; patient id no:1655185; CD4 antibody count:760; and number of cells/well: 0.4 x 10^6).

Fig. 3 is a graph showing the stimulatory effect of treatment according to the invention in lymphocytes of HIV-infected patients, compared to treatment using two control compounds (date of experiment: 3/15/95; patient id no: 1227604; CD4 antibody count: 230; number of cells/well: 0.16 x 10^6 ; and ½ area of a 96 well plate).

Fig. 4 is a graph showing the stimulatory effect of treatment according to the invention in lymphocytes of HIV-infected patients, compared to treatment using two control compounds (date of experiment 3/23/95; patient id no. 1586496; CD4 antibody count: 830; number of cells/well: 0.4 x $10^{6)}$.

Fig. 5 is a graph illustrating a stimulatory effect of an inhibitor according to the invention on PBMC *in vitro*, showing the correlation with CD4$^+$ counts. The data are plotted as the natural log of the stimulation index (vertical dimension) versus the natural log of the CD4$^+$ count of the patient (horizontal dimension)(71 patients total; P=<0.0001; RR=2.04 (1.5-2.9)).

Fig. 6 is a histogram demonstrating that an inhibitor according to the invention induces dose-dependent apoptosis in resting T-cells (these dosages are higher than the extremely low

doses used according to the invention). CD19+B cells and CD4+/CD8+Tcells were isolated (>90% and >97% purity, respectively). The cells were then incubated overnight in the presence or absence of VBBP (10^{-4} M or 10^{-6} M). The amount of death induced by VPB treatment was determined by 7AAD flow cytometry analysis. The data represents the mean percentage of death from duplicate samples.

Fig. 7 is a histogram demonstrating that an inhibitor according to the invention induces, at higher doses than in the invention, dose-dependent apoptosis in both $CD26^+$ and $CD26^-$ populations of PBMC. The $CD26^+$ and $CD26^-$ PBMC populations were found to be equally susceptible to DPPIV inhibitor induced death. PBMC were stained with the anti-CD26 monoclonal antibody, 4 EL, and then sorted into $CD26^+$ and $CD26^-$ populations using a facstar plus dual lasar flow cytometry. The cells expressing the highest level (5%) of CD26 and the cells expressing the lowest level (bottom 10%) of CD26 were isolated as the $CD26^+$ and $CD26^-$ populations respectively. The purity of the populations as examined by staining with the anti-CD26 monoclonal antibody, 134-2C2, is >90%. The $CD26^+$ and $CD26^-$ populations were cultured overnight in the presence or absence of various concentrations of VBP. The amount of death induced by VBP treatment was determined by 7AAD flow cytometry analysis. Data represent mean of death from duplicate samples +/- SD.

Fig. 8 is a graph showing that an inhibitor of CD26 (val-boroPro) inhibited the cytoplasmic enzyme as well.

Fig. 9 is a graph showing the stimulatory effect of treatment according to the invention in lymphocytes of HIV-infected patients, compared to treatment using two control compounds. Fluoroolefins did not induce cell death. PBMC were cultured overnight in the presence or absence of DPPIV inhibitors, L125, a fluoroolefin containing Npeptidal Oacil hydroxyl amine inhibitor or VBP. The amount of death induced was determined by 7AAD flow cytometry analysis. The data represents the mean percent death from duplicate samples.

Detailed Description

Therapeutic Compounds

Any organic compound can be used according to the invention which exhibits the following properties: (1) it is capable of crossing the membrane of human T-cells to reach the cytoplasm, where the compound can (2) interact with the cytoplasmic dipeptidase present in the T-cells, in order to (3) stimulate activation/proliferation of T-cells (and most preferably $CD4^+$ cells or CTLs) at concentrations below 10^{-8}M.

A simple screening method is described below for the identification of compounds which are candidate therapeutic compounds according to the invention.

Substrate and Enzyme Preparation

The first step is to provide a cytoplasmic enzyme preparation. The preparation need not be a pure enzyme sample; a crude cytoplasmic extract is sufficient to screen compounds for the desired activity. The extract can be prepared from any human T-cell line which is negative for CD26; an example of such a suitable cell line is the commercially available Jurkat cell line.

A suitable enzyme-containing cell extract can be prepared as follows. First, Jurkat cells (10^6-10^{11} cells) are grown and a cell pellet is obtained by centrifugation. The cell pellet is stored in frozen condition.

For use in the assay, the frozen pellet is thawed by the addition of ice cold lysis buffer, in the amount of approximately 1 ml per 10^8 cells. The liquefied material is homogenized with ten strokes of a Dounce homogenizer, and then clarified by centrifugation at 1500 g. The supernatant is removed (and saved), and the 1500 g pellet is resuspended in lysis buffer and homogenized with ten strokes of a Dounce homogenizer. Clarification is again carried out by centrifugation at 1500 g, 4°C.

The 1500 g supernatants are then combined, and EDTA is added to 5 mM. The resultant liquid is centrifuged at 75,000 g at 4°C for twenty minutes, and the supernatant is then removed and centrifuged at 175,000 g at 4°C for 60 minutes. The resultant supernatant, containing the cytosolic extract, is the DPPV activity-containing preparation used in the assay, described below, for candidate therapeutic compounds of the invention.

The assay is based on our observation that the T-cell cytoplasmic enzyme of interest is a post-prolyl cleaving serine protease. We therefore chose as a reporter substrate a compound which contains proline in the penultimate position; any of a number of substrates meeting this requirement can be used. In the assay described herein, we employed a fluorescent cleavage assay using the substrate Ala-ProAFC. Alternatively, a colorimetric assay can be carried out using as a substrate Gly-Pro-pNA. The choice of terminal amino acid is not critical, provided that the substrate contain a free terminal amino group.

In the assay we carried out, we employed a fluorescence spectrometer for excitation at 400 nm and emission at 505 nm. The spectrometer was calibrated for fluorescence intensity of 0.000= 10 mM HEPES, pH 7.4; and fluorescence intensity of 1.000= 10mM HEPES, 1 μM

AFC.

To carry out the assay, between 10 and 100 µl of enzyme extract, above, is diluted to 1 ml with 10mM HEPES, pH 7.4, containing 10mM Ala-ProAFC. At least one extract/substrate sample is run without test compound, to provide a standard for comparison with the test sample.

In the test samples, multiple samples are run containing varying concentrations, down to 10^{-8}M, of the test compound. The sample (with or without test compound) is placed in a cuvette, and inserted into a fluorescent spectrometer. Enzymatic activity is measured as the accumulation of fluorescence intensity (i.e., substrate cleavage product) over time (1 min.). A compound is identified as an inhibitor if the accumulative fluorescence is decreased as a result of the presence of the inhibiting compound.

Once a compound has been identified as an enzymatic inhibitor, as described above, further assays are carried out to determine whether the compound is capable of moving across the T-cell membrane into the cytoplasm; this is an assay which can be carried out using well-known techniques.

If desired, additional *in vitro* assays can be carried out using candidate compounds of the invention, prior to their use *in vivo*. One such assay employs the candidate compound at a very low concentration, in a test designed to determine whether at low concentrations the compound can stimulate the proliferation of PBMC from HIV-infected patients *in vitro*. As is shown in the data of Fig. 4, stimulation can be measured by, e.g., incorporation of a labeled nucleotide.

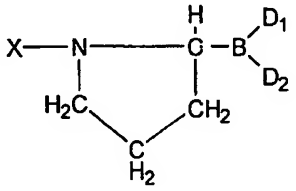
The compounds can also be tested at higher doses to determine whether they exhibit the opposite effect of proliferation, as above, i.e., dose-dependent apoptosis caused by enzyme inhibition, as in the experiments of Fig. 6.

Candidate Compounds

As is discussed above, compounds which are potentially capable of apoptosis induction at high doses and proliferation induction at low doses are those which, at normal or high doses, inhibit cytoplasmic T-cell dipeptidase, and can cross the T-cell membrane into the T-cell cytoplasm, where the enzyme interaction occurs. The compounds thus should be organic compounds which have a free amino group at the amino terminus; a proline or proline analog at the penultimate position; and an enzyme binding site which mimics the post-prolyl cleavage

site of cytoplasmic dipeptidase.

A number of known classes of compounds can be screened and used according to the invention. One such class are CD26 (i.e., DPPIV) inhibitors, including those described in Bachovchin et al., U.S. Patent No. 4,935,493, hereby incorporated by reference. In the '493 patent, there are described compounds having the structure:



where each D_1 and D_2, independently, is a hydroxyl group or a group which is capable of being hydrolyzed to a hydroxyl group in aqueous solution at physiological pH; and X comprises an amino acid or a peptide which mimics the site of the substrate recognized by a post-prolyl cleaving enzyme.

The compounds in the '493 patent are inhibitors of CD26, and are also candidate inhibitors of the invention. As is discussed above, because of the low concentrations of compounds used according to the invention, it is acceptable to use, in the invention, a compound which interacts not only with the cytoplasmic enzyme, but also CD26.

The class of compounds described in the '493 patent are also discussed and exemplified in Takacs et al., U.S. Patent Application Serial No. 07/923,337, corresponding to PCT Application No. WO94/03055, hereby incorporated by reference. In this application, one of the families of molecules in the '493 patent is described as the "Xaa-boroPro molecules," exemplified by Ala-boroPro, Pro-boroPro, and Gly-boroPro. These Xaa-boroPro molecules are all candidate compounds for use in the methods of the present invention. Two of these compounds are used in some of the examples described below; those compounds are Lys-boroPro ("KPB") and Val-boroPro ("VBP").

EXAMPLE 1

Peripheral blood mononuclear cells (PBMC) were obtained by standard methods from HIV-infected individuals, and from uninfected individuals. Varying dosages of KBP or VBP were contacted with the PBMC *in vitro*, and stimulation of proliferation was measured by

incorporation of ^3H thymidine (cpm). The results of these experiments are shown in Fig. 1: very low doses of the Val-boroPro and Lys-boroPro stimulated proliferation of PBMC from HIV-infected patients, but not PBMC from uninfected patients.

As shown in Fig. 1, at no concentration of the boroPro enzyme inhibitor did it affect the PBMC from uninfected individuals. The inhibitor, at moderate concentrations, also did not cause proliferation of PBMC from HIV-infected individuals, but it did cause marked proliferation at very low concentrations (10^{-9} and 10^{-10}M). These results are consistent with our hypothesis, discussed above, that, at low concentrations, these enzyme inhibitors exhibit a paradoxical effect: rather than inhibiting the apoptosis-controlling cytoplasmic T-cell enzyme, they interact with that enzyme in a manner which blocks inactivation and causes proliferation of T-cells.

Concordant results are shown in Fig. 2, a histogram showing that low doses of Lys-boroPro and Val-boroPro cause proliferation of PBMC of HIV-infected patients, while higher doses (10^{-4}M) do not have this effect.

The same results are shown in Figs. 3, 4, 9, and 10 which also present data for two control compounds OKT3, and PHA, both of which are non-specific mitogens.

Referring to Fig. 5, data are presented in a form which shows that low concentrations of the inhibitors of the invention have little effect on the PBMC of HIV-infected patients whose $CD4^+$ counts are lower than about 400 (the clinical indication for AIDS). In the graph of Fig. 5, the natural log of the stimulation index (the vertical axis) is plotted against the natural log of the $CD4^+$ count of the patients; as shown, above a count of 400 there is particularly significant stimulation of proliferation.

Fig. 6 is a graph demonstrating that purified T-cells are highly sensitive to cytoplasmic T-cell dipeptidase inhibitors in moderate concentrations. $CD19^+$B cells and $CD4^+/CD8^+$ T-cells were isolated to high purity and incubated overnight in Val-boroPro. The amount of cell death was determined by 7AAD flow cytometry analysis. Data represent % of cell death from duplicate samples. These data are consistent with our hypothesis that the inhibitors, in moderate concentration, inhibit a cytoplasmic enzyme which ordinarily protects against apoptosis.

Fig. 7 presents data demonstrating that $CD26^+$ and $CD26^-$ PBMC are equally susceptible to T-cell cytoplasmic enzyme inhibitor-induced death, where the inhibitor is administered immoderate concentrations. $CD26^+$ and $CD26^-$ populations were incubated

overnight in the presence or absence of various concentrations of Val-boroPro. The amount of cell death was determined by 7AAD flow cytometry analysis. Data represent mean % of death from duplicate samples. These data indicate that apoptosis-inhibiting T-cell cytoplasmic enzyme is present in both $CD26^+$ and $CD26^-$ T-cells.

Fig. 8 presents data showing the effects of an inhibitor useful in the invention, Val-boroPro. The experiments were carried out using two preparations: purified DPPIV (i.e., CD26), and Jurkat T-cell cytoplasmic extract, described above (Jurkat cells contain the cytoplasmic T-cell enzyme, but do not bear CD26 on their surfaces). These preparations were incubated with varying concentrations of Val-boroPro, and enzymatic activity was determined by measuring the accumulation of the fluorescent cleavage product of 7-amino-4-trifluoromethylcoumarin (AFC) released from the substrate Ala-ProAFC upon enzymatic cleavage. Val-boroPro inhibited both the enzyme DPPIV and the cytoplasmic T-cell enzyme in the Jurkat preparation.

Other embodiments are within the following claims.

What is claimed is:

CLAIMS

1. A method for stimulating proliferation of T-cells of a human patient suffering from a disease state characterized by the inability of said patient's T-cells to respond normally to T-cell proliferation-inducing stimuli; said method comprising contacting said T-cells, *in vitro* or *in vivo*, with an organic compound at an *in vitro* concentration below 10^{-8}M, or an *in vivo* blood concentration below 10^{-8}M, wherein said compound is characterized in that it binds to the post-prolyl cleaving dipeptidase activity present in the cytoplasm of Jurkat cells.

2. The method of claim 1, wherein said disease state is caused by HIV infection.

3. The method of claim 1, wherein said compound is further characterized in that, at a concentration of 10^{-4}M, it inhibits the cytoplasmic post-prolyl cleaving dipeptidase activity found in Jurkat T-cells.

4. The method of claim 3, wherein said compound is further characterized in that, at a concentration of 10^{-8}M, it binds to the cytoplasmic post-prolyl cleaving dipeptidase activity of $CD4^+$ T-cells of HIV-infected patients, to stimulate proliferation of said cells.

5. The method of claim 1, wherein said patient is infected with HIV, and said compound is administered to said patient to bring about a blood concentration of said compound below 10^{-10}M.

6. The method of claim 5, wherein said compound is further characterized in that it is capable of crossing the membrane of human $CD4^+$ T-cells to enter the cytoplasm.

7. A method for stimulating proliferation of T-cells which contain cytoplasmic post-prolyl cleaving dipeptidase activity and which are further characterized by the inability to respond normally to T-cell proliferation-inducing stimuli, said method comprising contacting said T-cells with an organic compound at a concentration below 10^{-8}M, wherein said compound is characterized in that:

(a) it is capable of crossing the membrane of said T-cells to enter the cytoplasm;

(b) it binds to said dipeptidase activity at a concentration below 10^{-8}M, and thus,

(c) stimulates proliferation of said T-cells at said concentration.

8. The method of claim 7, wherein said T-cells are $CD4^+$ cells.

9. The method of claim 8, wherein said compound enhances the ability of said $CD4^+$ T-cells to proliferate in response to antigenic stimulation.

10. The method of claim 1, wherein said compound is a serine protease inhibitor.

11. The method of claim 1, wherein said compound is administered to an HIV-infected patient.

12. The method of claim 10, wherein said serine protease inhibitor has a cleavage site or a binding site which mimics a post-proline serine protease cleavage site.

13. A method for testing a compound for enzyme inhibitory activity, said method comprising the steps of:

(a) providing a post-prolyl cleaving dipeptidase activity-containing cytoplasmic extract from T-cells which lack CD26 on their surfaces;

(b) contacting said extract with a serine protease reporter substrate of said dipeptidase activity, and with said compound; and

(c) determining whether said compound inhibits cleavage of said reporter substrate.

14. A method of treating a patient infected with HIV, said method comprising administering to said patient an organic compound characterized in that:

(a) it inhibits T-cell cytoplasmic post-prolyl cleaving dipeptidase activity at a concentration above 10^{-3}M;

(b) it interacts with said dipeptidase activity at a concentration below 10^{-8}M, enhancing the ability of said activity to inhibit apoptosis of T-cells of said patient; and

(c) it is capable of crossing the membrane of T-cells of said patient to enter the cytoplasm,

wherein said compound is administered to said patient so that its concentration in the

blood of said patient does not exceed 10^{-8}M.

15. The method of claim 14, wherein the $CD4^+$ count of said patient is higher than 400.

16. The method of claim 14, wherein said compound is administered in conjunction with a therapeutic agent which increases the $CD4^+$ count of HIV-infected patients.

17. A method of treating a viral infection in a patient, said method comprising administering to said patient a viral antigen, together with an adjuvant-acting amount of a compound characterized in that:

(a) it inhibits T-cell cytoplasmic post-prolyl dipeptidase activity at a concentration of about 10^{-5}M;

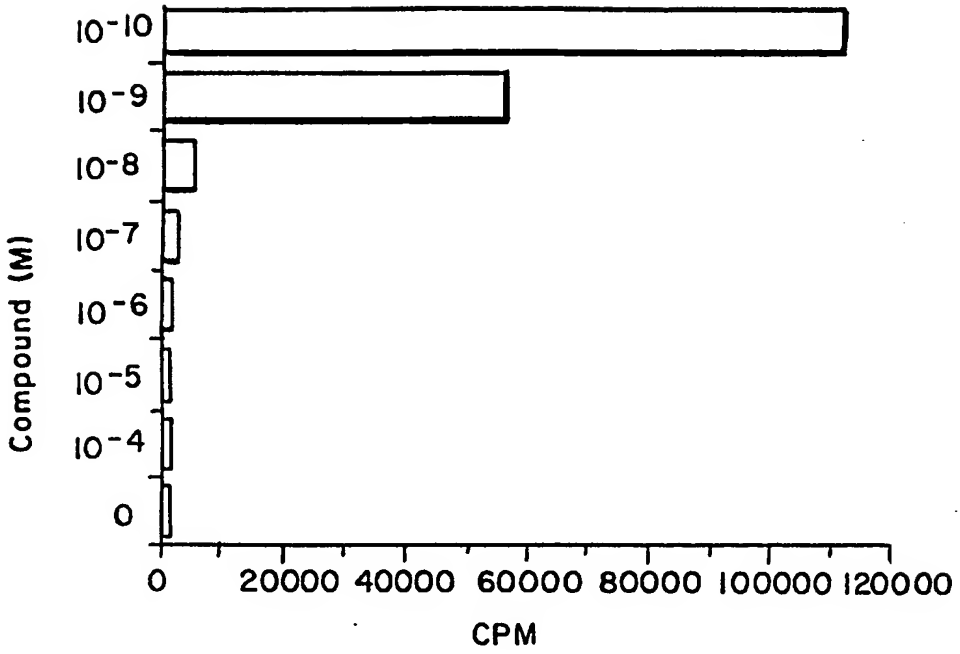
(b) it interacts with said dipeptidase activity at a concentration below 10^{-8}M, enhancing the ability of said activity to inhibit apoptosis of T-cells of said patient; and

(c) it is capable of crossing the membrane of T-cells of said patient to enter the T-cell cytoplasm,

wherein said compound is administered so that its concentration in the blood of said patient does not exceed 10^{-8}M.

18. The method of claim 11, wherein said virus is HIV.

19. The method of claim 17, wherein said compound is administered orally.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80000
100000
120000
CPM

FIG. 1A

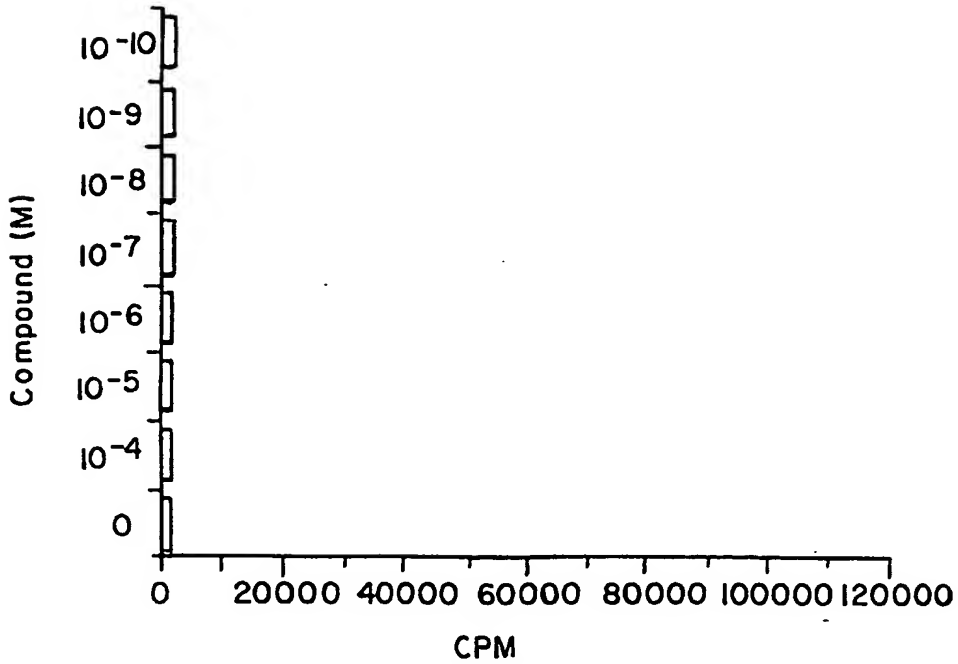
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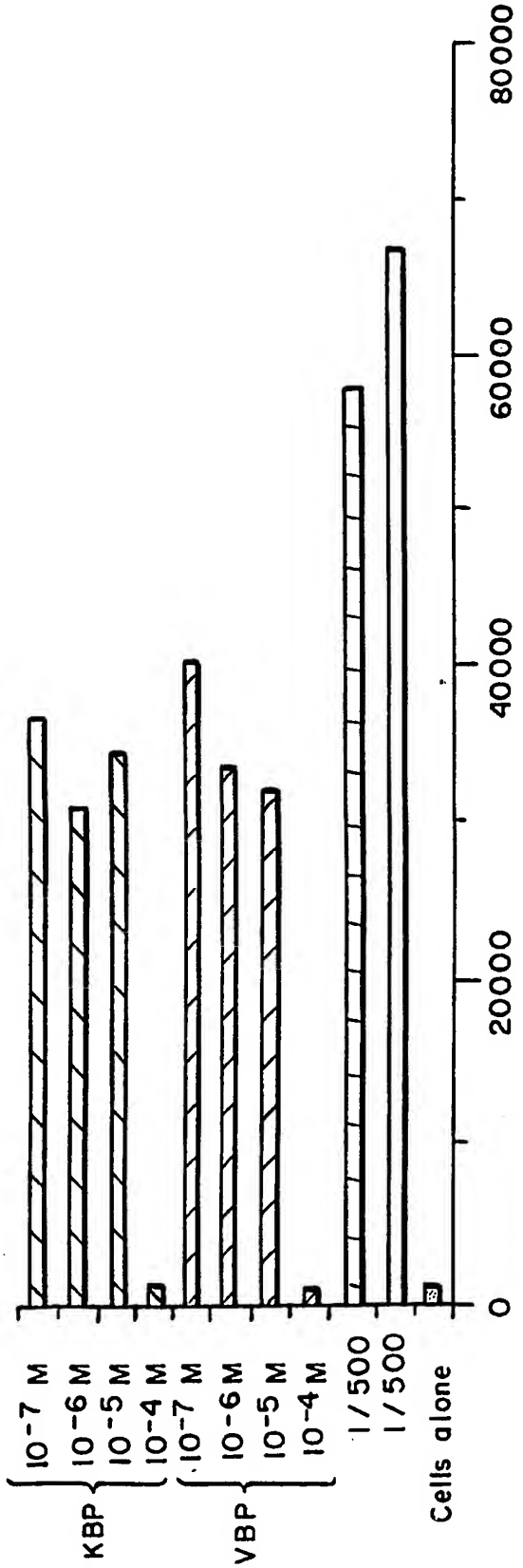
FIG. 1B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60000
40000
20000
0
CPM
10⁻⁷ M
10⁻⁶ M
10⁻⁵ M
10⁻⁴ M
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10⁻⁵ M
10⁻⁴ M
1/500
1/500
Cells alone
KBP
VBP

FIG. 2

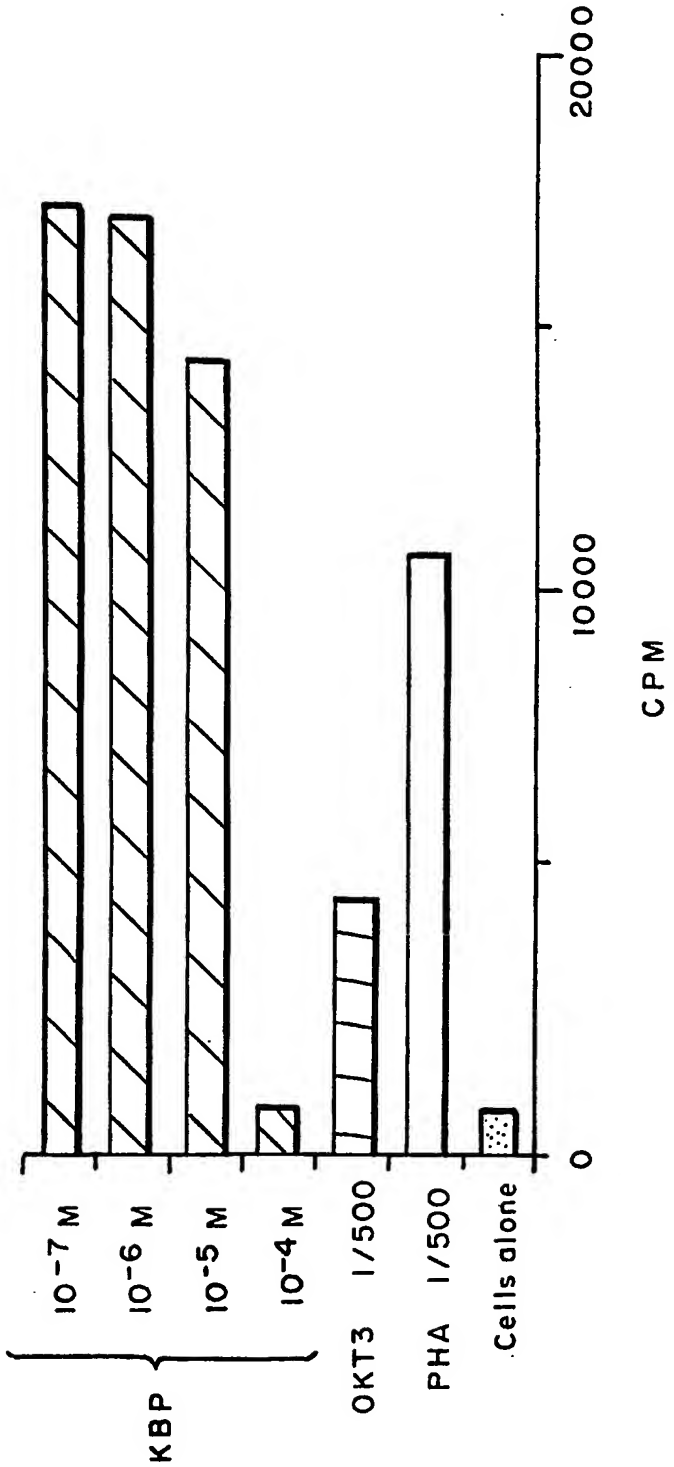
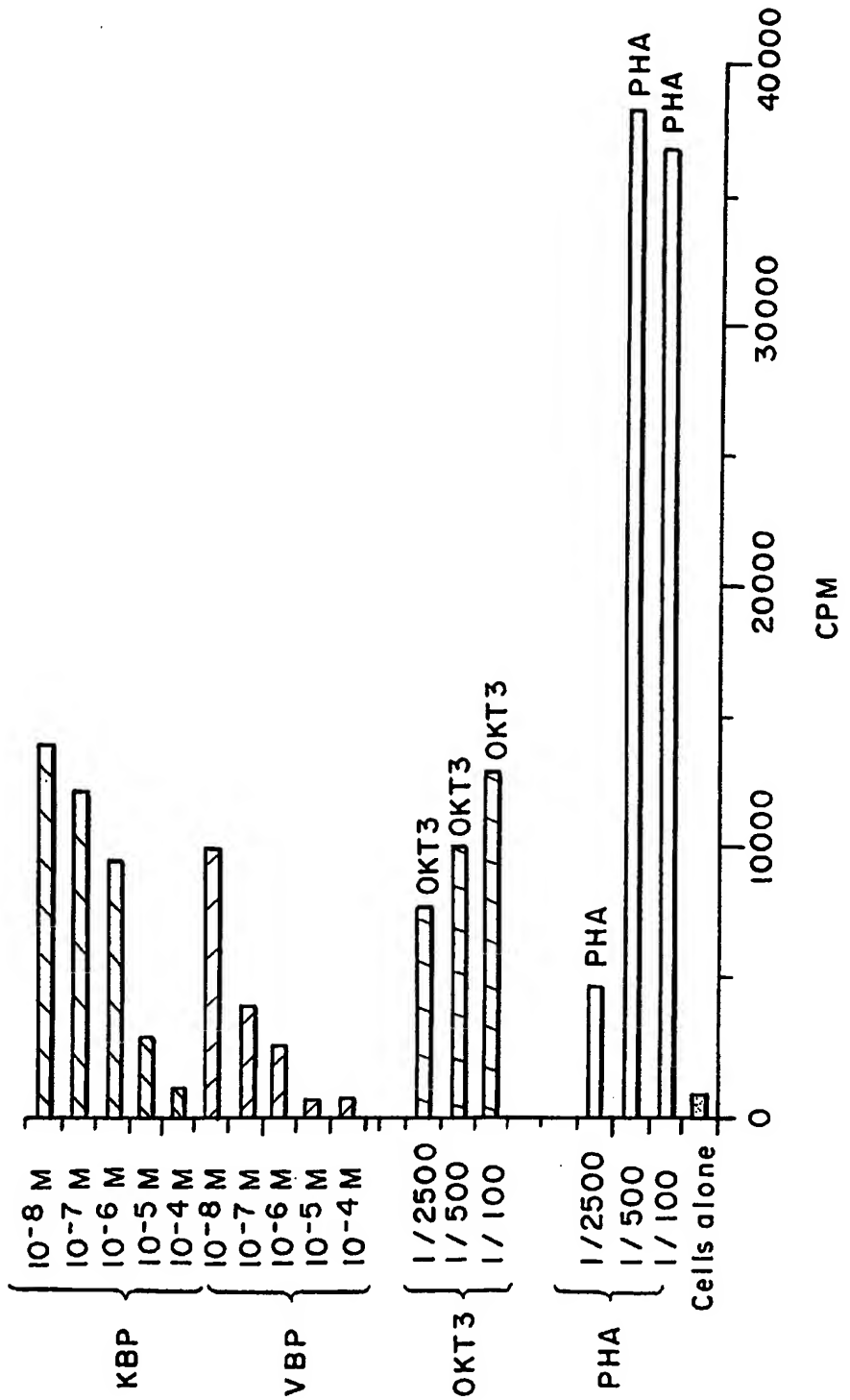

20000
10000
0
CPM
KBP
10⁻⁷ M
10⁻⁶ M
10⁻⁵ M
10⁻⁴ M
OKT3 1/500
PHA 1/500
Cells alone

FIG. 3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1/500
1/100
1/2500
1/500
1/100
Cells alone
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OKT3
PHA
PHA
PHA
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30000
40000
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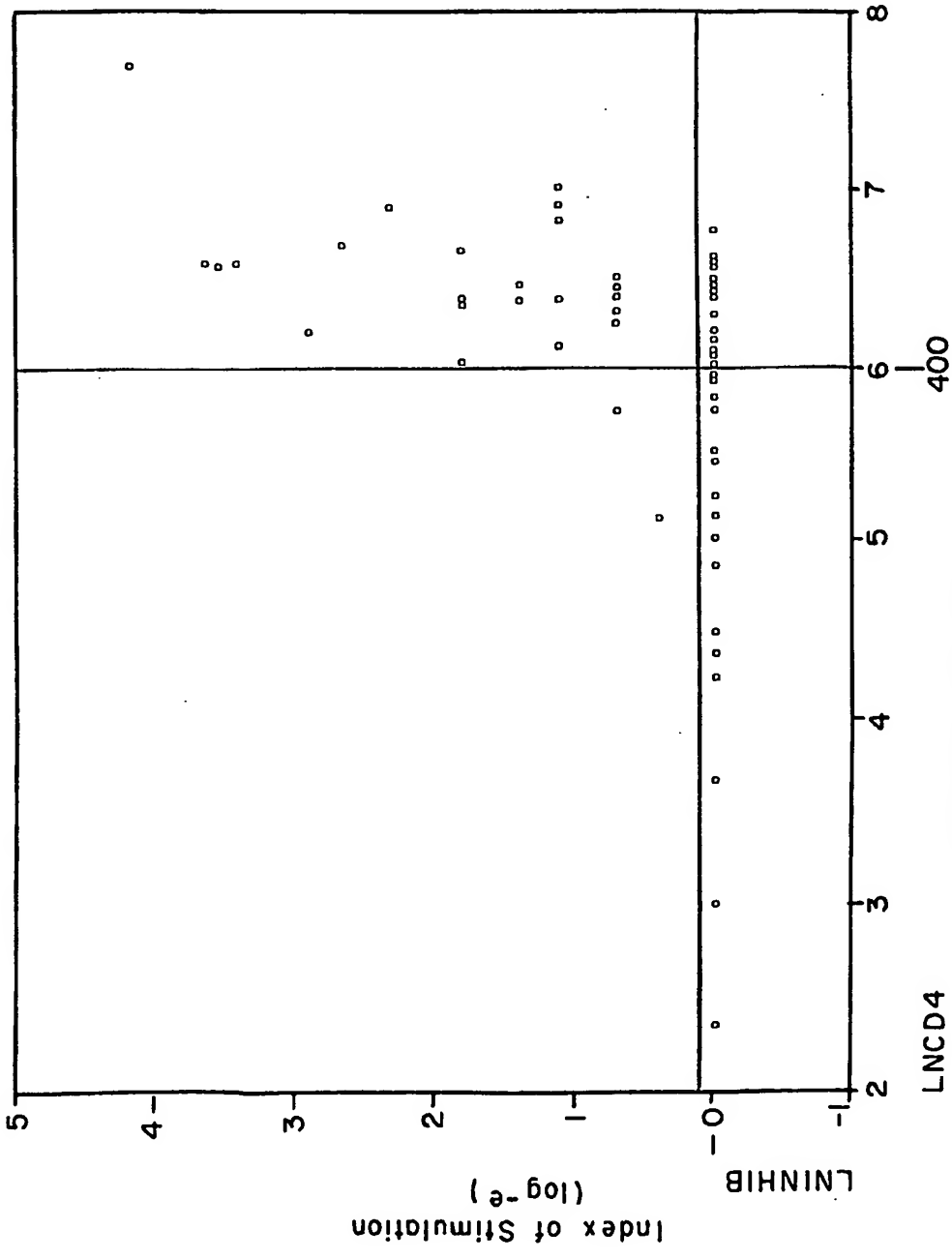
FIG. 4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(log-e)
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3
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0
-1
2
3
4
5
6
7
8
LNCD4
400
Absolute CD4 Count
(log-e)

FIG. 5

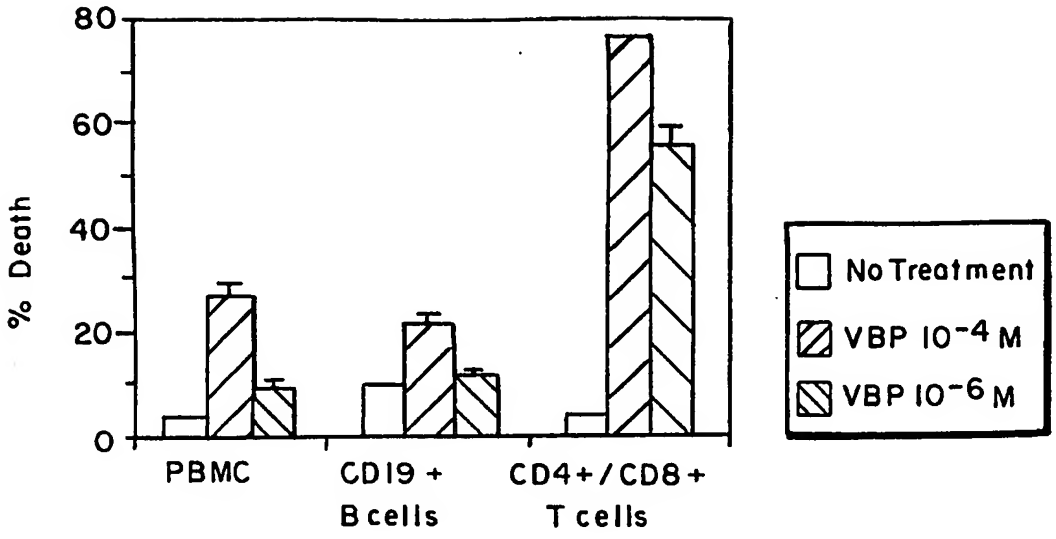
80
60
40
20
0
% Death
PBMC
CD19 +
B cells
CD4+/CD8+
T cells
No Treatment
VBP 10-4 M
VBP 10-6 M

FIG.6

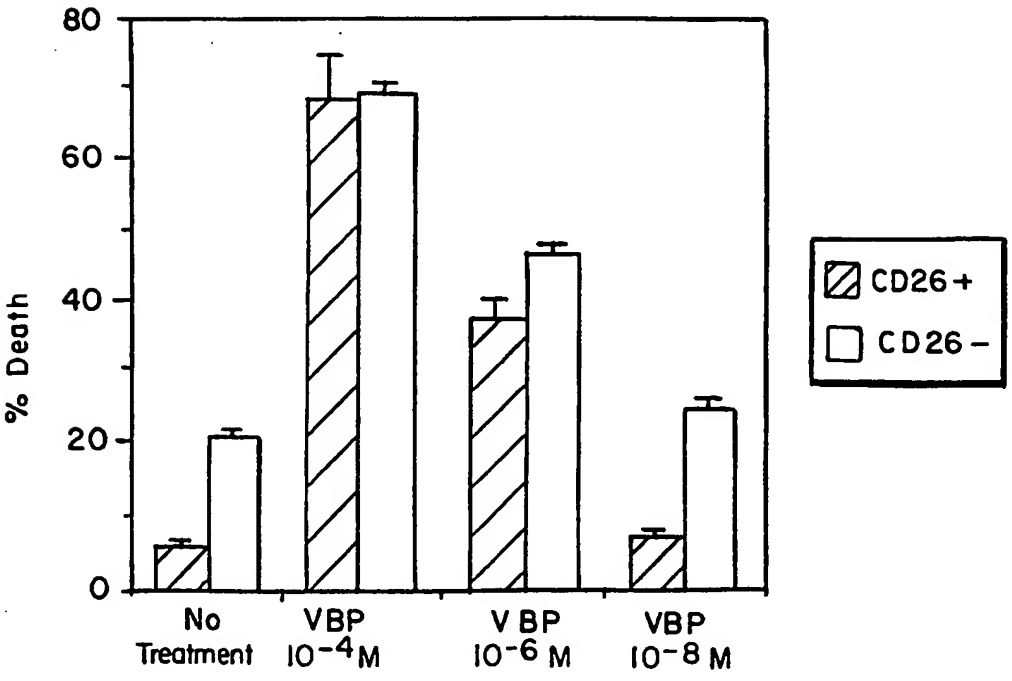
80
60
40
20
0
% Death
No
Treatment
VBP
10-4M
VBP
10-6 M
VBP
10-8 M
CD26+
CD26-

FIG.7

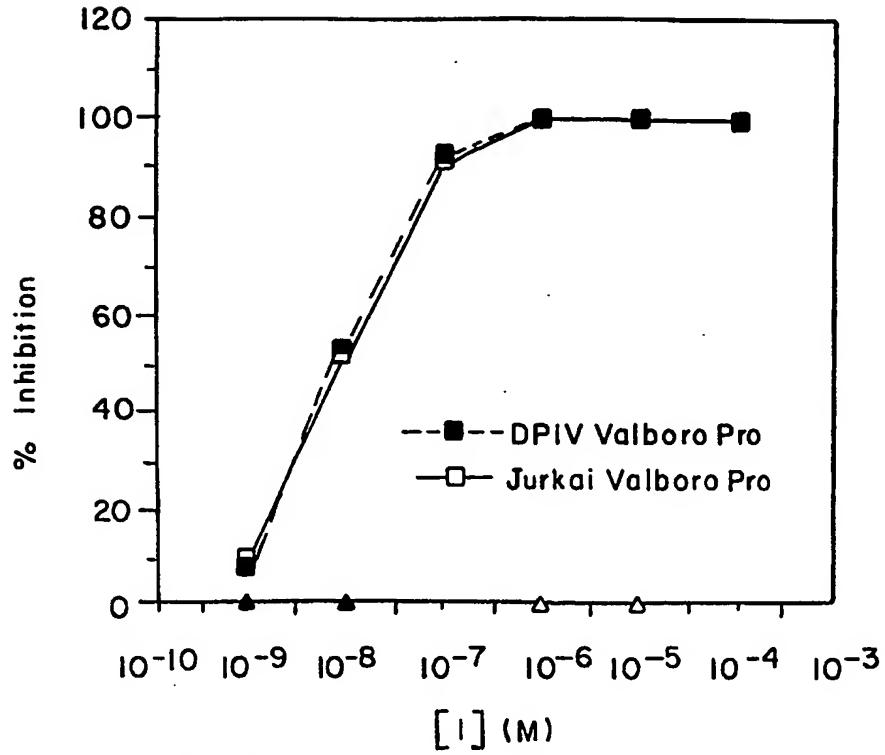
120
100
80
60
40
20
0
% Inhibition
DPIV Valboro Pro
Jurkai Valboro Pro
10-10 10-9 10-8 10-7 10-6 10-5 10-4 10-3
[I] (M)

FIG. 8

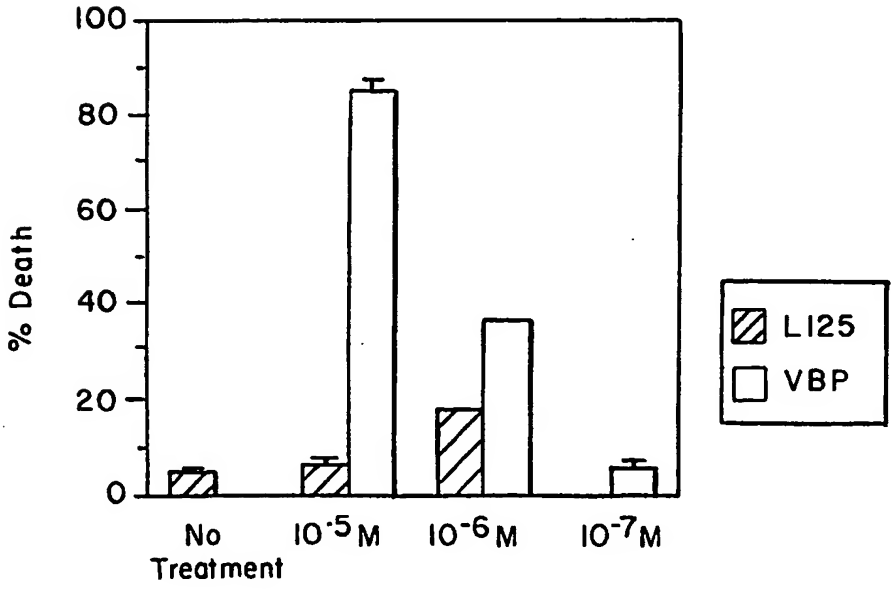
100
80
60
40
20
0
% Death
L125
VBP
No Treatment
10-5M
10-6M
10-7M

FIG. 9

INTERNATIONAL SEARCH REPORT

In tional Application No
PCT/US 98/08838

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/55 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 03055 A (US HEALTH ;UNIV TUFTS (US)) 17 February 1994 see page 4, line 9 - page 6, line 27 see page 8 - page 9; example 4 ---	1-19
A	WO 91 17767 A (NEW ENGLAND MEDICAL CENTER INC ;UNIV TUFTS (US)) 28 November 1991 see page 1, line 16 - page 3, line 11 --- -/--	1-19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 August 1998	13/08/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sitch, W

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

In .tional Application No
PCT/US 98/08838

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	FLENTKE ET AL: "INHIBITION OF DIPEPTIDYL AMINOPEPTIDASE IV (DP-IV) BY XAA-BOROPRO DIPEPTIDES AND USE OF THESE INHIBITORS TO EXAMINE THE ROLE OF DP-IV IN T-CELL FUNCTION" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA, vol. 88, 1991, pages 1556-1559, XP000578025 see page 1556 see abstract	1-19

1

INTERNATIONAL SEARCH REPORT

..ternational application No.
PCT/US 98/ 08838

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 1-4,7-10,12 partially and 5,6,11,14-19 completely is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
PCT/US 98/08838

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9403055 A	17-02-1994	AU 4794393 A	03-03-1994
WO 9117767 A	28-11-1991	NONE	